



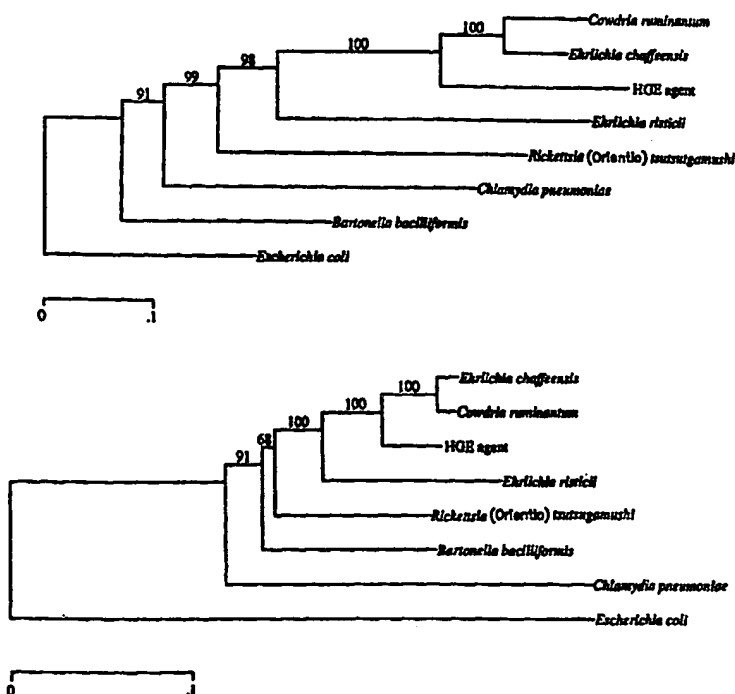
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(54) Title: METHODS TO DETECT GRANULOCYTIC EHRLICHIOSIS

(57) Abstract

An isolated nucleic acid molecule associated with human granulocytic ehrlichiosis is provided. Also provided are methods to detect the presence of the nucleic acid molecule, and antibodies specific for the polypeptide encoded by the nucleic acid molecule, in a sample derived from a mammal.



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METHODS TO DETECT GRANULOCYTIC EHRLICHIOSIS

Background of the Invention

Human granulocytic ehrlichiosis (HGE) is a recently described illness caused by a bacterial species that is phylogenetically similar to, or conspecific with, an ehrlichial genogroup comprising the veterinary pathogens *Ehrlichia equi* and *E. phagocytophilia* (Chen et al., J. Clin. Micro., 32, 589 (1994); Dumler et al., J. Clin. Micro., 33, 1098 (1995)). The only vectors demonstrated to transmit the HGE agent are *Ixodes* sp. ticks, although anecdotal evidence suggests that the American dog tick (*Dermacentor variabilis*) may also be implicated in HGE transmission. HGE typically presents as an acute febrile illness with headache, myalgias, leukopenia, thrombocytopenia, and elevated levels of alkaline phosphatase, lactate dehydrogenase, and aminotransferases (Bakken et al., JAMA, 272, 212 (1994); Bakken et al., JAMA, 275, 199 (1996); Pancholi et al., J. Infect. Dis., 172, 1007 (1995); Telford et al., Proc. Natl. Acad. Sci USA, 93, 6209 (1996)). Severe cases, when left untreated, may be fatal.

For the direct recovery of the HGE agent from clinical specimens, mouse inoculation, molecular detection, or cultivation of the organism in HL60 cells have been used. However, the duration of bacteremia with the HGE agent is not known, so the reliability of these tests beyond the acute stage of the illness has not been established. Antibody tests based on seroconversion to *E. equi* antigen have been used to identify infected persons after the initial stage of the illness (Bakken et al., JAMA, 272, 212 (1994); Bakken et al., JAMA, 275, 199 (1996)). However, the indirect fluorescent antibody (IFA) assay based on *E. equi* antigen is labor intensive, not widely available, and the interpretation of results is often subject to substantial individual variation (Bakken et al., JAMA, 275, 199 (1996); Dumler et al., *supra*; Dumler et al., Dermat. Clinics, 12, 25 (1994)).

Thus, what is needed is an improved method to detect the presence of an infectious agent associated with HGE.

Summary of the Invention

The present invention is based upon the direct recovery (or isolation) of a gene encoding an immunogenic polypeptide associated with granulocytic ehrlichiosis in humans from patient tissues. Direct recovery of genes encoding immunodominant antigens from infected tissues, i.e., without prior growth in culture, is particularly useful to prepare immunodiagnostic reagents for emerging, uncultured, or difficult to culture, pathogens, e.g., the agents associated with human granulocytic ehrlichiosis (HGE), *Tropheryma whippelii*, the bacillary agent associated with Whipple's disease, and other organisms related to mycobacteria which are yet to be cultivated *in vitro*. Moreover, direct recovery of DNA comprising open reading frames can also facilitate development of customized immunodiagnostic reagents, even when recovery of the infectious agent is not possible or when extensive agent variability (i.e., "quasispecies") precludes development of universal immunodiagnostic reagents.

Thus, the invention provides an isolated and purified DNA molecule comprising a preselected DNA sequence encoding an immunogenic polypeptide, a biologically active subunit, or a biologically active variant thereof, the presence of which is associated with granulocytic ehrlichiosis in a mammal, e.g., in humans. Preferably, the preselected DNA sequence encodes an immunogenic polypeptide which is specifically associated with the agent which causes human granulocytic ehrlichiosis (HGE). A preferred embodiment of the invention provides a preselected DNA sequence which encodes an immunogenic polypeptide having an amino acid sequence comprising SEQ ID NO:2, e.g., the preselected DNA sequence comprises SEQ ID NO:1. The invention further provides an isolated and purified DNA molecule which is complementary to SEQ ID NO:1.

The invention also provides an expression cassette comprising a preselected DNA sequence operably linked to a promoter functional in a host cell wherein said DNA sequence encodes an immunogenic polypeptide, a biologically active variant or subunit thereof, wherein said polypeptide is

associated with granulocytic ehrlichiosis in a mammal. The expression of the preselected DNA sequence in host cells yields a polypeptide which is an ehrlichial antigen. Preferably, the expression cassette of the invention comprises a preselected DNA sequence encoding an immunogenic fusion polypeptide, a portion of which comprises an ehrlichial-specific polypeptide. The remainder of the DNA sequence preferably encodes a polypeptide that enhances the immunogenicity of the fusion polypeptide *in vivo*.

As used herein, "an immunogenic polypeptide associated with granulocytic ehrlichiosis" is preferably a polypeptide having the amino acid sequence comprising SEQ ID NO:2, as well as variants of SEQ ID NO:2 which have at least about 80%, preferably at least about 90%, and more preferably at least about 95%, identity or homology to SEQ ID NO:2, or a biologically active subunit thereof. Biologically active subunits of the immunogenic polypeptide of the invention, and biologically active variants of the immunogenic polypeptide of the invention and subunits thereof, falling within the scope of the invention have at least about 10%, preferably at least about 50%, and more preferably at least about 90%, the activity of the polypeptide comprising SEQ ID NO:2. The activity of an immunogenic polypeptide of the invention can be measured by methods well known to the art including, but not limited to, the ability of the polypeptide to be bound by antibodies specific for the infectious agent associated with granulocytic ehrlichiosis (see Example II), or the ability of the polypeptide to elicit a sequence-specific immunologic response when the polypeptide is administered to an organism, e.g., a mammal such as rabbit, goat, sheep, rat or mouse. Preferably, the immunologic response is a humoral response, i.e., antibody response, directed to a particular epitope on the polypeptide. More preferably, the presence of antibodies specific for that epitope correlates with the granulocytic ehrlichiosis infection status of the organism.

Further provided is an isolated and purified immunogenic polypeptide, a biologically active subunit or variant thereof, which is associated with granulocytic ehrlichiosis in a mammal. The polypeptide is useful to detect the presence of antibodies in mammals, e.g., deer, mice, rats, dogs and humans,

which are specific for an infectious agent associated with granulocytic ehrlichiosis. Preferably, the polypeptide has an amino acid sequence comprising SEQ ID NO:2. The isolated and purified polypeptides of the invention are useful to prepare an immunogenic composition, such as a vaccine comprising the
5 polypeptide, in combination with a pharmaceutically acceptable carrier, wherein the administration of the immunogenic composition or vaccine to a mammal induces the production of antibodies to the polypeptide. In particular, an immunogenic composition or vaccine which comprises a polypeptide having an epitope which is specific for the agent associated with human granulocytic
10 ehrlichiosis is preferred.

The invention provides a method of expressing a nucleic acid molecule encoding an immunogenic polypeptide, the presence of which is associated with granulocytic ehrlichiosis, in a cultured host cell stably transformed with a chimeric vector comprising said nucleic acid molecule
15 operably linked to control sequences recognized by the host cell transformed with the vector, and recovering the polypeptide from the host cell.

The invention further provides a diagnostic method comprising contacting an amount of DNA obtained from a physiological sample which comprises cells from a mammal at risk of, or afflicted with, granulocytic
20 ehrlichiosis, with an amount of at least two complementary oligonucleotides under conditions effective to amplify the DNA by a polymerase chain reaction so as to yield an amount of amplified DNA. At least one oligonucleotide binds specifically to a DNA sequence encoding a polypeptide derived or isolated from an infectious agent associated with granulocytic ehrlichiosis. The presence of
25 the amplified DNA is then detected or determined. The presence of the amplified DNA is indicative of a mammal at risk of, or afflicted with, granulocytic ehrlichiosis. The results described below show that an amplified DNA was detected using DNA obtained from blood of two humans afflicted with granulocytic ehrlichiosis. Sequence and phylogenetic analysis of the
30 polypeptide encoded by the amplified DNA shows that the polypeptide is homologous to the *E. coli* heat shock protein-60 (HSP60) and related to, but

distinct from, a homologous protein found in *E. chaffeensis* (Sumner et al., Infection & Immunity, 61, 3546 (1993)) and from other rickettsia-like organisms known to infect humans.

Also provided is a method for detecting DNA encoding an
5 immunogenic polypeptide associated with granulocytic ehrlichiosis in a mammal. The method comprises isolating or deriving an amount of DNA from a mammalian physiological sample which comprises cells suspected of containing DNA encoding the immunogenic polypeptide. The DNA is contacted with an amount of at least two oligonucleotides under conditions effective to amplify the
10 DNA by a polymerase chain reaction so as to yield an amount of amplified DNA. At least one oligonucleotide is specific for the DNA encoding the immunogenic polypeptide. The presence of the amplified DNA is then determined or detected.

As used herein, the term an oligonucleotide "specific for the DNA
15 encoding a polypeptide from an infectious agent associated with granulocytic ehrlichiosis" or "specific for DNA encoding an immunogenic polypeptide associated with granulocytic ehrlichiosis" means a DNA sequence that has at least about 80%, more preferably at least about 90%, and more preferably at least about 95%, sequence identity or homology to SEQ ID NO:1. An oligonucleotide
20 or primer of the invention has at least about 7-50, preferably at least about 10-40, and more preferably at least about 15-35, nucleotides. Preferably, the oligonucleotide primers of the invention comprise at least 7 nucleotides at the 3' of the oligonucleotide primer which have at least about 80%, more preferably at least about 85%, and more preferably at least about 90%, identity to SEQ ID
25 NO:1. Thus, the oligonucleotides of the invention may also include sequences which are unrelated to nucleic acid sequences of an infectious agent associated with granulocytic ehrlichiosis, e.g., they may encode restriction endonuclease recognition sequences. Preferred oligonucleotides of the invention include an oligonucleotide comprising SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ
30 ID NO:8, SEQ ID NO:9 or SEQ ID NO:10.

Also provided is a diagnostic kit for detecting DNA which encodes at least a portion of a polypeptide specific to an infectious agent associated with granulocytic ehrlichiosis in a physiological sample suspected of containing said DNA. The kit comprises packaging containing, separately
5 packaged, (a) a known amount of a first oligonucleotide, wherein the oligonucleotide consists of at least about 7-50 nucleotides, and wherein the oligonucleotide has at least about 80% identity to SEQ ID NO:1, and (b) a known amount of a second oligonucleotide, wherein the oligonucleotide consists of at least about 7-50 nucleotides, and wherein the oligonucleotide has at least
10 about 80% identity to a nucleotide sequence which is complementary to SEQ ID NO:1.

Thus, the invention also provides an oligonucleotide which consists of at least about 7-50 nucleotides and which has at least about 80% identity to, or has complementarity to a nucleotide sequence having, SEQ ID
15 NO:1.

Also provided is a method for detecting or determining the presence of antibodies in a mammalian physiological sample, which antibodies are specific for an infectious agent that is associated with granulocytic ehrlichiosis. The method comprises contacting an amount of purified
20 polypeptide with the sample which is suspected of comprising antibodies specific for the infectious agent, for a sufficient time to form binary complexes between at least a portion of the antibodies and a portion of the purified polypeptide. The polypeptide is one which is expressed by, and is encoded by the nucleic acid of, the infectious agent. The presence or amount of the
25 complexes is then determined or detected. The results reported hereinbelow demonstrated that a portion of purified polypeptide, expressed *in vitro* from a DNA molecule of the invention, is bound by antibodies from mice experimentally infected with the agent associated with human granulocytic ehrlichiosis. Thus, the detection of antibody responses to this and other
30 ehrlichial antigens can be used in ELISA-based immunoassays for the serodiagnosis of granulocytic ehrlichial infection in animals and in humans.

Moreover, the results show that the expression of a gene encoding an antigenic polypeptide, wherein the gene is obtained by direct recovery from a clinical sample without the benefit of prior biological amplification via cultivation *in vitro*, is useful to detect a humoral immune response to an infectious agent, i.e., the agent associated with human granulocytic ehrlichiosis.

Further provided is a method for detecting granulocytic ehrlichiosis in a mammal. The method comprises contacting an amount of purified polypeptide with a sample suspected of containing antibodies to said polypeptide, for a sufficient time to form binary complexes between at least a portion of the antibodies and a portion of the purified polypeptide. The polypeptide is one which is encoded by the nucleic acid from an infectious agent associated with granulocytic ehrlichiosis. The presence or amount of the binary complexes is then determined or detected. The presence of said complexes is indicative of a mammal at risk of, or afflicted with, granulocytic ehrlichiosis.

Also provided is a diagnostic kit for detecting or determining antibodies that specifically react with an infectious agent which is associated with granulocytic ehrlichiosis. The kit comprises packaging, containing, separately packaged, a solid phase capable of binding a polypeptide and a known amount of a purified polypeptide, the presence of which is associated with granulocytic ehrlichiosis. Preferably, the polypeptide has an amino acid sequence comprising SEQ ID NO:2.

Brief Description of the Figures

Figure 1. Nucleotide sequence (SEQ ID NO:1) encoding the heat shock protein-60 (HSP60 or groEL) homolog of the human granulocytic ehrlichiosis (HGE) agent.

Figure 2. Alignment of the predicted amino acid sequence for the HSP60 homologue encoded by a nucleic acid sequence associated with HGE (SEQ ID NO:2) and the HSP60 polypeptide encoded by *E. chaffeensis* DNA (SEQ ID NO:3).

Figure 3. Neighbor-joining analysis of DNA sequences from several of the species having homology with the HGE agent-derived HSP60

homologue. Phylogenetic analysis was based on A) HSP60 gene sequences and B) 16S rRNA gene sequences. The bootstrap replicate percentages appear above each branch. The scale is provided as a measurement of the relative phylogenetic distance.

5 Figure 4. Agarose gel and Southern blot analysis of PCR
amplification products from Balb/C mouse blood and HL60 cells infected with
the HGE agent. DNA isolated from Balb/C mouse blood and HL60 cells was
subjected to PCR using primers Ehr521 and Ehr790. Lane 1, 100 bp molecular
weight marker. Lanes 2-4, amplified DNA from the blood of Balb/C mice which
10 were infected with a midwest strain (WI-1) of the HGE agent by syringe
inoculation. Lane 5, amplified DNA from HL60 cells infected with a midwest
strain (WI-1) of the HGE agent. Lane 6, blank. Lane 7, uninfected Balb/C
mouse control. Lane 8, uninfected HL60 control. Lane 9, no DNA control.
Lane 10, 100 bp molecular weight marker.

15 Figure 5. Detection of murine serum antibody response to the
HGE agent HSP60-like recombinant antigen.

A) Sera from Balb/C mice infected for 28 days by intraperitoneal
inoculation with a midwest strain (WI-1) of the HGE agent or with *B.*
burgdorferi strain N40. Lanes 1-2, uninfected mice. Lanes 3-5, mice infected
20 by syringe inoculation with the HGE agent. Lanes 6-8, mice infected by syringe
inoculation with 10^3 *B. burgdorferi* strain N40. Lane 9, uninfected mouse. *E.*
equi antibody titers are indicated above each lane.

B) Reactivity of mouse sera from Balb/C mice (see panel A) for
glutathione-S transferase (GST). All lanes were incubated with anti-GST
25 primary antibodies. Lane 1, recombinant GST-HGE HSP-60 fusion polypeptide.
Lanes 2-3, sera from uninfected mice. Lanes 4-6, sera from mice infected with
the HGE agent. Lanes 7-9, sera mice infected with *B. burgdorferi* strain N40.
Lane 10, sera from an uninfected mouse. Lanes 11-12, blocking agent alone.
Lane 13, 29 kD GST polypeptide.

30 C) Sera from C3H/HeJ mice infected by tick transmission with an east
coast strain (NCH-1) of the HGE agent or with *B. burgdorferi* strain N40. Lanes

1-2, sera from mice infected with the HGE agent for six months. Lane 3, sera from a mouse that was fed upon by *Ixodes* sp. ticks infected with the NCH-1 strain of the HGE agent but was negative by blood smear and antibody response. Lanes 4-5, sera from mice infected for 19 months with the HGE agent. Lanes 6-9, sera from mice infected for six months with the HGE agent. Lane 10, sera from a mouse infected for less than one month with the HGE agent. Lane 11, sera from a mouse that was fed upon by *Ixodes* sp. ticks infected with the NCH-1 strain of the HGE agent but was negative by blood smear and antibody response. Lanes 12-14, sera from mice that were fed upon by uninfected *Ixodes* sp. ticks. Lanes 15-16, sera from mice infected for 10 months with *B. burgdorferi* strain N40. *E. equi* antibody titers are indicated above each lane.

Detailed Description of the Invention

In order to better detect the infectious agent which causes, or is associated with, granulocytic ehrlichiosis, and in particular human granulocytic ehrlichiosis (HGE), granulocytic ehrlichiosis agent-specific DNA which encodes an immunogenic polypeptide is isolated from patients with the disease, or is isolated from non-human mammals or cell lines experimentally infected with the agent. Besides identifying the presence of infectious agent-specific nucleic acid sequences, or antibodies which bind to the polypeptide encoded by those nucleic acid sequences, in a mammal, the identification and isolation of these nucleic acid sequences or polypeptides may be useful to provide a clinically useful diagnostic test, or in molecular-based therapeutics.

Once a correlation has been established between the presence of the nucleic acid sequence, or antibodies to the polypeptide encoded by that sequence, and a particular disease, such as granulocytic ehrlichiosis or monocytic ehrlichiosis, mammalian physiological samples, e.g., tissue biopsies, blood sera or plasma, can be analyzed for the presence of the nucleic acid sequence, e.g., by an amplification reaction such as the polymerase chain reaction, or by screening sera for the presence of antibodies specific for the purified form of the polypeptide. The presence or amount of the nucleic acid

molecule or antibodies in the mammalian sample can be compared to a control sample from a disease-free mammal.

An isolated and purified DNA molecule which encodes a polypeptide specific to the infectious agent can also be employed in an expression cassette to synthesize agent-specific polypeptides *in vitro*. *In vitro* prepared agent-specific polypeptide can be employed to prepare polyclonal or monoclonal antibodies specific for the agent, or in assays useful to detect the presence of agent-specific antisera in mammals. *In vitro* synthesized polypeptide may also be employed in a vaccine or immunogenic composition which, when administered to a mammal, can elicit antibodies which can inhibit or block subsequent infection of the host by the agent.

The DNA sequences (molecules) of the present invention are also useful to detect the replication or presence of the infectious agent in infected samples, to detect related DNA molecules and to amplify nucleic acid sequences, wherein said sequences can fall within the scope of the present invention.

Sources of Nucleic Acids Encoding an Immunogenic Polypeptide from the Granulocytic Ehrlichiosis Agent

Sources of nucleotide sequences from which the present DNA molecules can be derived include nucleic acid from any mammalian cellular source, known or believed to be naturally or experimentally infected by a granulocytic ehrlichiosis, preferably a human granulocytic ehrlichiosis, causative agent, preferably derived from blood cells or cell lines derived therefrom, from which nucleic acid molecules can be isolated by methods well known to the art.

Isolation of a Gene Encoding a Granulocytic Ehrlichiosis-Specific Immunogenic Polypeptide

A nucleic acid molecule encoding an immunogenic, granulocytic ehrlichiosis agent-specific polypeptide, can be identified and isolated using standard methods, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY (1989). For example, polymerase

chain reaction (PCR) can be employed to isolate and clone HGE agent-specific genes. "PCR" refers to a procedure or technique in which amounts of a preselected fragment of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195.

5 In PCR, sequence information from the ends of the region of interest or beyond is employed to identify and synthesize oligonucleotide primers comprising at least 7-8 nucleotides. These primers will be identical or similar in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total
10 genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, and the like. See generally Mullis et al., Cold Spring Harbor Symp. Quant. Biol., 51, 263 (1987); Erhlich, ed., PCR Technology, (Stockton Press, NY, 1989).

 Thus, primers are made to correspond to DNA encoding highly
15 conserved regions of polypeptides or to nucleotide sequences which were identified and compared to generate the primers, e.g., by a sequence comparison of other bacterial HSP60 genes. At least two primers are prepared, one of which is predicted to anneal to the antisense strand, and preferably one of which is predicted to anneal to the sense strand of a DNA molecule which encodes the
20 immunogenic polypeptide. The products of each PCR reaction are separated via an agarose gel and all consistently amplified products can be gel-purified and cloned directly into a suitable vector, such as a plasmid vector. The resultant plasmids are subjected to restriction endonuclease and dideoxy sequencing of double-stranded plasmid DNAs.

25 Another approach to identify, isolate and clone genes which encode a polypeptide associated with the granulocytic ehrlichiosis causative agent is to screen a cDNA library generated from infected tissues, e.g., formed from DNA from HGE agent-infected blood cells. Screening for DNA fragments that encode all or a portion of a gene encoding an immunogenic, granulocytic
30 ehrlichiosis agent-specific polypeptide can be accomplished by probing the library with a probe, which comprises sequences that are highly conserved

between genes believed to be related to the gene encoding the immunogenic polypeptide, e.g., HSP60 gene probe, or by screening of plaques for binding to antibodies from a mammal that has been infected by the granulocytic ehrlichiosis agent. DNA fragments that bind to the probe, or which are immunoreactive with
5 antibodies, can be subcloned into a suitable vector and sequenced and/or used as probes to identify other cDNA or genomic sequences encoding all or a portion of the immunogenic polypeptide.

As used herein with respect to a DNA molecule or polypeptide, the terms "isolated and/or purified" refer to *in vitro* isolation of a DNA or
10 polypeptide molecule from its natural cellular environment, and from association with other components of the cell, such as nucleic acid or protein, so that it can be sequenced, replicated, and/or expressed. For example, "isolated granulocytic ehrlichiosis agent-specific nucleic acid" is RNA or DNA containing greater than 7, preferably 15, and more preferably 20 or more, sequential nucleotide bases.
15 The sequential nucleotide bases encode a biologically active granulocytic ehrlichiosis, preferably an HGE, agent-specific polypeptide or a fragment thereof, or a biologically active variant granulocytic ehrlichiosis, preferably an HGE, agent-specific polypeptide or a fragment thereof. The DNA or RNA is complementary to the non-coding strand, or complementary to the coding strand,
20 of RNA or DNA from the agent which causes, or is associated with, granulocytic ehrlichiosis and not complementary to the RNA or DNA from related organisms which do not cause, or are not associated with, granulocytic ehrlichiosis, or hybridizes to said RNA or DNA and remains stably bound under stringent conditions.

25 Thus, the RNA or DNA is "isolated" in that it is free from at least one contaminating nucleic acid with which it is normally associated in the natural source of the RNA or DNA, and the isolated RNA or DNA is preferably substantially free of any other mammalian RNA or DNA. The phrase "free from at least one contaminating source of nucleic acid with which it is normally
30 associated" includes the case where the nucleic acid is reintroduced into the source or natural cell but is in a different chromosomal location or is otherwise

flanked by nucleic acid sequences not normally found in the source cell. An example of isolated HGE agent-specific nucleic acid is RNA or DNA that encodes a biologically active, i.e., immunogenic, HGE agent-specific polypeptide sharing at least about 80%, preferably at least about 90%, sequence identity with the HGE agent-specific polypeptide of Figure 2.

As used herein, the term "recombinant nucleic acid" or "preselected nucleic acid," e.g., "recombinant DNA sequence or segment" or "preselected DNA sequence or segment" refers to a nucleic acid, i.e., to DNA that has been derived or isolated from any appropriate tissue source, that may be subsequently chemically altered *in vitro*, so that its sequence is not naturally occurring, or corresponds to naturally occurring sequences that are not positioned as they would be positioned in a genome which has not been transformed with exogenous DNA. An example of preselected DNA "derived" from a source, would be a DNA sequence that is identified as a useful fragment within a given organism, and which is then chemically synthesized in essentially pure form. An example of such DNA "isolated" from a source would be a useful DNA sequence that is excised or removed from said source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering.

Thus, recovery or isolation of a given fragment of DNA from a restriction digest can employ separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. See Lawn et al., Nucleic Acids Res., 9, 6103 (1981), and Goeddel et al., Nucleic Acids Res., 8, 4057 (1980). Therefore, "preselected DNA" includes completely synthetic DNA sequences, semi-synthetic DNA sequences, DNA sequences isolated from biological sources, and DNA sequences derived from RNA, as well as mixtures thereof.

As used herein, the term "derived" with respect to a RNA molecule means that the RNA molecule has complementary sequence identity to a particular DNA molecule.

5 Variants of the DNA Molecules of the Invention

Nucleic acid molecules encoding amino acid sequence variants of an granulocytic ehrlichiosis agent-specific immunogenic polypeptide, e.g., HSP60, can be prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the
10 case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of a granulocytic ehrlichiosis agent-specific polypeptide.

Oligonucleotide-mediated mutagenesis is a preferred method for
15 preparing amino acid substitution variants of the granulocytic ehrlichiosis agent-specific polypeptide. This technique is well known in the art as described by Adelman et al., DNA, 2, 183 (1983). Briefly, granulocytic ehrlichiosis agent-specific DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a
20 plasmid or bacteriophage containing the unaltered or native DNA sequence encoding a granulocytic ehrlichiosis agent-specific polypeptide. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the DNA
25 encoding the granulocytic ehrlichiosis agent-specific polypeptide.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize
30 properly to the single-stranded DNA template molecule. The oligonucleotides

are readily synthesized using techniques known in the art such as that described by Crea et al., Proc. Natl. Acad. Sci. U.S.A., 75, 5765 (1978).

The DNA template can be generated by those vectors that are either derived from bacteriophage M13 vectors (the commercially available
5 M13mp18 and M13mp19 vectors are suitable), or those vectors that contain a single-stranded phage origin of replication as described by Viera et al., Meth. Enzymol., 153, 3 (1987). Thus, the DNA that is to be mutated may be inserted into one of these vectors to generate single-stranded template. Production of the single-stranded template is described in Sections 4.21-4.41 of Sambrook et al.,
10 Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, N.Y. 1989). Alternatively, single-stranded DNA template may be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino
15 acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus
20 formed so that one strand of DNA encodes the mutated form of the granulocytic ehrlichiosis agent-specific polypeptide, and the other strand (the original template) encodes the native, unaltered sequence of the granulocytic ehrlichiosis agent-specific polypeptide. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After the cells
25 are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for polypeptide production, generally an expression vector of the type typically employed for transformation of an
30 appropriate host.

The method described immediately above may be modified so that a homoduplex molecule is created wherein both strands of the plasmid contain the mutations(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above.

- 5 A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thiodeoxyribocytosine called dCTP-(aS) (which can be obtained from the Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a
- 10 strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

- After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be
- 15 digested with ExoIII nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This
- 20 homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101.

- A preferred embodiment of the invention is an isolated and purified DNA molecule comprising a preselected DNA sequence encoding a granulocytic ehrlichiosis agent-specific HSP60 polypeptide comprising SEQ ID
- 25 NO:2, wherein the DNA segment comprises SEQ ID NO:1. Variants of SEQ ID NO:2 having nucleotide substitutions which are "silent" are also within the scope of the invention. That is, when nucleotide substitutions are present in a codon, the same amino acid is encoded by the codon with the nucleotide substitution as is encoded by the codon without the substitution. For example, leucine is
- 30 encoded by the codons TTA, TTG, CTT, CTC, CTA and CTG. A variant of SEQ ID NO:1 at the eleventh codon in the mature polypeptide (TTA in SEQ ID

NO:1) includes the substitution of TTG for TTA. Other "silent" nucleotide substitutions in SEQ ID NO:1 which can encode a polypeptide having SEQ ID NO:2 can be ascertained by reference to page D1 in Appendix D in Sambrook et al., Molecular Cloning: A Laboratory Manual (1989). Nucleotide substitutions
5 can be introduced into DNA segments by methods well known to the art. See, for example, Sambrook et al., *supra*.

Chimeric Expression Cassettes

To prepare recombinant polypeptides useful to detect antibodies
10 which bind to the granulocytic ehrlichiosis agent, an expression cassette is prepared comprising a preselected DNA sequence which encodes a granulocytic ehrlichiosis agent-specific immunogenic polypeptide operably linked to a promoter. Preferably, the immunogenic polypeptide is a fusion polypeptide.

Generally, the expression cassette is in the form of chimeric
15 DNA, and comprises plasmid DNA that can also contain coding regions flanked by control sequences which promote the expression of the preselected DNA segment once the expression cassette is introduced into a host cell. As used herein, "chimeric" means that a vector comprises DNA from at least two different species, or comprises DNA from the same species, which is linked or
20 associated in a manner which does not occur in the "native" or wild type of the species. Aside from preselected DNA sequences that serve as transcription units for granulocytic ehrlichiosis agent-specific polypeptides or portions thereof, a portion of the preselected DNA may be untranscribed, serving a regulatory or a structural function. For example, the preselected DNA may itself comprise a
25 promoter that is active in bacterial, fungal, insect or mammalian cells, or may utilize a promoter already present in the genome that is the transformation target. There are many promoter elements well known to the art that may be employed in the practice of the invention.

Other elements functional in the host cells, such as introns,
30 enhancers, polyadenylation sequences and the like, may also be a part of the preselected DNA. Such elements may or may not be necessary for the function

of the DNA, but may provide improved expression of the DNA by affecting transcription, stability of the mRNA, or the like. Such elements may be included in the DNA as desired to obtain the optimal performance of the transforming DNA in the cell.

5 "Control sequences" are defined to mean DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotic cells, for example, include a promoter, and optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters,
10 polyadenylation signals, and enhancers.

 "Operably linked" is defined to mean that the nucleic acids are placed in a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a prepolyptide that participates in the
15 secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader,
20 contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

 The preselected DNA to be introduced into the cells further will
25 generally contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of transformed cells from the population of cells sought to be transformed. Alternatively, the selectable marker may be carried on a separate piece of DNA and used in a co-transformation procedure. Both selectable markers and reporter genes may be flanked with appropriate
30 regulatory sequences to enable expression in the host cells. Useful selectable markers are well known in the art and include, for example, antibiotic and

herbicide-resistance genes, such as *neo*, *hpt*, *dhfr*, *bar*, *aroA*, *dapA* and the like. See also, the genes listed on Table 1 of Lundquist et al. (U.S. Patent No. 5,848,956).

- Reporter genes are used for identifying potentially transformed cells and for evaluating the functionality of regulatory sequences. Reporter genes which encode for easily assayable polypeptides are well known in the art. In general, a reporter gene is a gene which is not present in or expressed by the recipient organism or tissue and which encodes a polypeptide whose expression is manifested by some easily detectable property, e.g., enzymatic activity.
- Preferred genes include the chloramphenicol acetyl transferase gene (*cat*) from Tn9 of *E. coli*, the beta-glucuronidase gene (*gus*) of the *uidA* locus of *E. coli*, and the luciferase gene from firefly *Photinus pyralis*. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells.
- The general methods for constructing recombinant DNA which can transform target cells are well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the DNA useful herein. For example, J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (2d ed., 1989), provides suitable methods of construction.

Transformation into Host Cells

- The recombinant DNA can be readily introduced into host cells, e.g., mammalian, bacterial, fungal or insect cells by transfection with an expression vector comprising DNA encoding a granulocytic ehrlichiosis agent-specific polypeptide by any procedure useful for the introduction into a particular cell, e.g., calcium phosphate precipitation, lipofection, electroporation, and the like, to yield a transformed cell having the cDNA stably integrated into its genome, so that the DNA molecules, sequences, or segments, of the present invention are expressed by the host cell. That is, the present invention also provides a transformed host cell having a genome augmented by a recombinant

(non-native) DNA sequence, preferably by a chromosomally integrated recombinant (genetically engineered) DNA sequence that includes a gene encoding a granulocytic ehrlichiosis agent-specific polypeptide.

As used herein, the term "cell line" or "host cell" is intended to refer to well-characterized homogenous, biologically pure populations of cells. These cells may be eukaryotic cells that are neoplastic or which have been "immortalized" *in vitro* by methods known in the art, as well as primary cells, or prokaryotic cells. The cell line or host cell is preferably of prokaryotic origin, but cell lines or host cells of eukaryotic origin may be employed, including plant, insect, yeast, fungal or mammalian sources. Generally, the preselected DNA sequence is not resident in the genome of the uninfected host cell.

"Transfected" or "transformed" is used herein to include any host cell or cell line, the genome of which has been altered or augmented by the presence of at least one preselected DNA sequence, which DNA is also referred to in the art of genetic engineering as "heterologous DNA," "recombinant DNA," "exogenous DNA," "genetically engineered DNA," "non-native DNA," or "foreign DNA," wherein said DNA is isolated and introduced into the genome of the host cell or cell line by the process of genetic engineering. The host cells of the present invention are typically produced by transfection with a DNA sequence in a plasmid expression vector, a viral expression vector, or as an isolated linear DNA sequence.

Granulocytic Ehrlichiosis Agent-Specific Polypeptides

The present invention provides an isolated, purified granulocytic ehrlichiosis agent-specific polypeptide, which can be prepared by recombinant DNA methodologies. The general methods for isolating and purifying a recombinantly expressed polypeptide from a host cell are well known to those in the art. Examples of the isolation and purification of such polypeptides are given in Sambrook et al., cited *supra*. Moreover, since the present invention provides the amino acid sequence of an HGE agent-specific polypeptide (Figure 2), it or bioactive variants thereof can also be synthesized by the solid phase peptide

synthetic method. This established and widely used method, including the experimental procedures, is described in the following references: Stewart et al., Solid Phase Peptide Synthesis, W.H. Freeman Co., San Francisco (1969); Merrifield, J. Am. Chem. Soc., **85** 2149 (1963); Meienhofer in "Hormonal
5 Proteins and Peptides," ed.; C.H. Li, Vol. 2 (Academic Press, 1973), pp. 48-267; and Bavaay and Merrifield, "The Peptides," eds. E. Gross and F. Meienhofer, Vol. 2 (Academic Press, 1980) pp. 3-285.

When a granulocytic ehrlichiosis agent-specific polypeptide is expressed in a recombinant cell it is necessary to purify the polypeptide from
10 other recombinant cell proteins or polypeptides to obtain preparations that are substantially homogenous as to the granulocytic ehrlichiosis agent-specific polypeptide. For example, the culture medium or lysate can be centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The granulocytic ehrlichiosis agent-specific polypeptide may
15 then be purified from the soluble protein fraction. Alternatively, the granulocytic ehrlichiosis agent-specific polypeptide may be purified from the insoluble fraction, i.e., refractile bodies (see, for example, U.S. Patent No. 4,518,526). Granulocytic ehrlichiosis agent-specific polypeptide may be purified from contaminant soluble or membrane proteins and polypeptides by
20 fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on an anion-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; or ligand affinity chromatography, and the like.

25 If expressed as a fusion polypeptide, the fusion polypeptide may be purified by methods specific for the non-granulocytic ehrlichiosis agent-specific portion of the polypeptide. For example, if the fusion polypeptide is a glutathione-S transferase (GST) fusion polypeptide, GST 4B beads may be employed to purify the fusion polypeptide.

30 Granulocytic ehrlichiosis agent-specific polypeptide, variant granulocytic ehrlichiosis agent-specific polypeptide, or a biologically active

- subunit thereof can also be prepared by *in vitro* transcription and translation reactions. A granulocytic ehrlichiosis agent-specific expression cassette can be employed to generate granulocytic ehrlichiosis agent-specific transcripts which are subsequently translated *in vitro* so as to result in a preparation of
- 5 substantially homogenous granulocytic ehrlichiosis agent-specific polypeptide, variant granulocytic ehrlichiosis agent-specific polypeptide, or a biologically active subunit thereof. The construction of vectors for use *in vitro* transcription/translation reactions, as well as the methodologies for such reactions, are well known to the art.
- 10 Once isolated from the resulting transgenic host cells or from *in vitro* transcription/translation reactions, derivatives and chemically derived variants of the granulocytic ehrlichiosis agent-specific polypeptide can be readily prepared. For example, amides of the granulocytic ehrlichiosis agent-specific polypeptides of the present invention may also be prepared by
- 15 techniques well known in the art for converting a carboxylic acid group or precursor, to an amide. A preferred method for amide formation at the C-terminal carboxyl group is to cleave the polypeptide from a solid support with an appropriate amine, or to cleave in the presence of an alcohol, yielding an ester, followed by aminolysis with the desired amine.
- 20 Salts of carboxyl groups of the granulocytic ehrlichiosis agent-specific polypeptide may be prepared in the usual manner by contacting the peptide with one or more equivalents of a desired base such as, for example, a metallic hydroxide base, e.g., sodium hydroxide; a metal carbonate or bicarbonate base such as, for example, sodium carbonate or sodium bicarbonate;
- 25 or an amine base such as, for example, triethylamine, triethanolamine, and the like.
- N-acyl derivatives of an amino group of the present polypeptides may be prepared by utilizing an N-acyl protected amino acid for the final condensation, or by acylating a protected or unprotected peptide. O-acyl
- 30 derivatives may be prepared, for example, by acylation of a free hydroxy peptide or peptide resin. Either acylation may be carried out using standard acylating

reagents such as acyl halides, anhydrides, acyl imidazoles, and the like. Both N- and O-acylation may be carried out together, if desired. In addition, the internal granulocytic ehrlichiosis agent-specific amino acid sequence, for example the HGE agent-specific amino acid sequence of Figure 2, can be modified by

5 substituting one or two conservative amino acid substitutions for the positions specified, including substitutions which utilize the D rather than L form.

The invention also provides variant or modified forms of the granulocytic ehrlichiosis agent-specific polypeptide, e.g., the HGE agent-specific HSP60 polypeptide. One or more of the residues of this polypeptide can be

10 altered, so long as the variant polypeptide has at least about 10%, preferably at least about 50%, and more preferably at least about 90%, of the biological activity of the granulocytic ehrlichiosis polypeptide, e.g., the polypeptide having SEQ ID NO:2. Conservative amino acid substitutions are preferred--that is, for example, aspartic-glutamic as acidic amino acids; lysine/arginine/histidine as

15 basic amino acids; leucine/isoleucine, methionine/valine, alanine/valine as hydrophobic amino acids; serine/glycine/alanine/threonine as hydrophilic amino acids.

Acid addition salts of the polypeptides may be prepared by contacting the polypeptide with one or more equivalents of the desired inorganic

20 or organic acid, such as, for example, hydrochloric acid. Esters of carboxyl groups of the polypeptides may also be prepared by any of the usual methods known in the art.

Granulocytic Ehrlichiosis Agent-Specific Variant Polypeptides

25 Variant granulocytic ehrlichiosis agent-specific polypeptides will have at least one amino acid substitution relative to the polypeptide sequence of a particular granulocytic ehrlichiosis-agent isolate, e.g., SEQ ID NO:2. In particular, amino acids are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of exemplary

30 substitutions. More preferred substitutions are under the heading of preferred

substitutions. After the substitutions are introduced, the products are screened for biological activity.

TABLE 1

5	Original	Exemplary	Preferred
	Residue	Substitutions	Substitutions
10	Ala (A)	val; leu; ile	val
	Arg (R)	lys; gln; asn	lys
	Asn (N)	gln; his; lys; arg	gln
	Asp (D)	glu	glu
	Cys (C)	ser	ser
	Gln (Q)	asn	asn
	Glu (E)	asp	asp
	Gly (G)	pro	pro
	His (H)	asn; gln; lys; arg	arg
	Ile (I)	leu; val; met; ala; phe norleucine	leu
20	Leu (L)	norleucine; ile; val; met; ala; phe	ile
	Lys (K)	arg; gln; asn	arg
	Met (M)	leu; phe; ile	leu
	Phe (F)	leu; val; ile; ala	leu
	Pro (P)	gly	gly
	Ser (S)	thr	thr
	Thr (T)	ser	ser
	Trp (W)	tyr	tyr
	Tyr (Y)	trp; phe; thr; ser	phe
	Val (V)	ile; leu; met; phe; ala; norleucine	leu

Amino acid substitutions falling within the scope of the invention,
 30 are, in general, accomplished by selecting substitutions that do not differ
 significantly in their effect on maintaining (a) the structure of the polypeptide
 backbone in the area of the substitution, for example, as a sheet or helical
 conformation, (b) the charge or hydrophobicity of the molecule at the antigenic
 site, or (c) the bulk of the side chain. Naturally occurring residues are divided
 35 into groups based on common side-chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophilic: cys, ser, thr;
- (3) acidic: asp, glu;
- (4) basic: asn, gln, his, lys, arg;
- 5 (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic; trp, tyr, phe.

The invention also envisions granulocytic ehrlichiosis agent-specific polypeptide variants with non-conservative substitutions. Non-conservative substitutions entail exchanging a member of one of the classes
10 described above for another. Amino acid substitutions are introduced into the DNA molecules of the invention by methods well known to the art. For example, see the description hereinabove for the introduction of silent mutations into the DNA molecules of the invention.

15 Detection of Granulocytic Ehrlichiosis Agent-Specific DNA by Polymerase Chain Reaction (PCR)

To detect a DNA encoding a granulocytic ehrlichiosis agent-specific polypeptide, DNA is isolated from a cellular sample suspected of containing granulocytic ehrlichiosis agent associated DNA, e.g., DNA isolated
20 from a human suspected of having HGE. The DNA can be isolated by methods known to the art. See Sambrook et al., *supra*. The isolated DNA is mixed with primers specific for the granulocytic ehrlichiosis agent DNA so as to yield amplified, HGE agent-specific DNA product. Preferably, a hemi-nested PCR is employed. For example, primers such as BR588 (5' YGG ATA YCT TTC KCC
25 TTA YTT T 3'; SEQ ID NO:8), SP949 (5' CTT GGT ACT GCT AAG AGC GTG 3'; SEQ ID NO:9) or BR1212 (5' CCT TCC TCA ACA GCA GCT CTA 3'; SEQ ID NO:10) may be useful to detect HGE agent-specific sequences when employed in a hemi-nested PCR.

To detect the PCR amplified product, the reaction mixture is
30 typically subjected to agarose gel electrophoresis or another separation technique, and the presence or absence of the granulocytic ehrlichiosis agent-

specific amplified DNA is detected. Detection of the amplified granulocytic ehrlichiosis agent DNA may be accomplished by excising or eluting the fragment from the gel (for example, see Lawn et al., Nucleic Acids Res., 9, 6103 (1981), and Goeddel et al., Nucleic Acids Res., 8, 4057 (1980)), cloning the
5 amplified product into a cloning site of a suitable vector and sequencing the cloned insert. Alternatively, the granulocytic ehrlichiosis agent-amplified DNA may be detected using Southern hybridization with a granulocytic ehrlichiosis agent-specific oligonucleotide probe, or comparing its electrophoretic mobility with DNA standards of known molecular weight.

10

Uses of Recombinant Granulocytic Ehrlichiosis Agent-Specific Polypeptides

Once isolated, granulocytic ehrlichiosis agent-specific polypeptide and its antigenically active variants, derivatives and fragments thereof can be used in assays for the detection of the presence of the granulocytic
15 ehrlichiosis agent in samples derived from biological materials suspected of containing the polypeptide or anti-polypeptide antibodies. Thus, the polypeptide can be used as a "capture antigen" to bind to anti-polypeptide antibodies in a sample of a mammalian physiological fluid. For example, a physiological sample comprising antibodies is mixed with purified granulocytic ehrlichiosis
20 agent-specific polypeptide so as to yield a binary complex. The antibodies which are bound to the polypeptide are separated from the antibodies which are not bound to the polypeptide. Then the complex is detected or determined. Preferably, the complex is detected by an anti-mammal IgG antibody (sheep, goat, mouse, rat human etc.).

25 The invention will be further described with reference to the following Examples.

Example I

Isolation and sequence of the HSP60 (groEL) homologue from the HGE Agent

To provide an improved method to detect the infection of a
30 mammal by the HGE agent, the DNA sequences which encode a conserved polypeptide, i.e., the HSP60 protein, of organisms which are closely related to

the HGE agent, e.g., *E. chaffeensis* and *Cowdria ruminantium* (GenBank accession numbers L10917 and U13638, respectively), were aligned using the GAP algorithm of the Wisconsin package. From this alignment, several broad-range PCR primer sets were synthesized (Integrated DNA Technologies,

5 Coralville, IA) from regions conserved among the two genes (Table 2).

One hundred (100) pmol of primer HSP961 (SEQ ID NO:4) and HSP1754a (SEQ ID NO:5) (Table 2) were incorporated into a PCR master mix containing 10% glycerol, 1X PCR buffer II (Perkin Elmer Corp., Norwalk, CT), 2.0 mM MgCl₂ (Perkin Elmer Corp.), 200 μM each of dATP, dGTP, and dCTP,
10 100 μM each of dTTP and dUTP, 2.5 units of Amplitaq DNA polymerase (Perkin Elmer Corp.) and 5 μl of a patient DNA sample. Patient DNA was obtained by extracting DNA from whole blood samples from two human patients with the IsoQuick Nucleic Acid Extraction Kit (Orca Research Inc., Bothell, WA) as previously described (Kolbert, in: PCR Protocols for Emerging
15 Infectious Diseases, ASM Press, Washington, D.C. (1996)). The isolated DNA was stored at 4°C prior to amplification. The two patients were previously confirmed to have HGE by clinical presentation, antibody titer to *E. equi*, and by PCR analysis for a portion of the 16S rDNA gene.

Amplification was performed in a 100 μl reaction volume with a
20 Temptronic thermal cycler (Barnstead-Thermolyne, Dubuque, IA) using the following parameters: 94°C incubation for 4 minutes, then 40 cycles at 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 45 seconds, followed by a 72°C incubation for 5 minutes. Subsequent to PCR amplification, a 10 μl aliquot of each amplification product was electrophoresed in a 2% agarose gel (Seakem
25 GTG, FMC Bioproducts, Rockland, ME) to assess amplification integrity and the remainder was purified (Qiaquick PCR purification kit, Qiagen Inc., Chatsworth, CA) in preparation for DNA sequencing.

PCR amplification of each of the two patient DNA samples produced an 820 bp product. The amplicon was sequenced on an ABI 373 or
30 ABI 377 automated DNA sequencing instrument (Perkin Elmer Applied Biosystems Division, Foster City, CA) and the consensus sequence was then

used as a query in the GenBank sequence database in order to identify homologous sequences (FASTA algorithm of the Wisconsin package, Genetics Computer Group, Program Manual, 8th ed.). The sequences which were most closely related to the query sequence were the HSP60 homologs from *E.*

- 5 *chaffeensis* and *C. ruminantium* with 76.5% and 76.3% nucleotide sequence identity, respectively.

Table 2. Nucleotide sequence and function of oligonucleotide primers used for recovery and sequencing HSP60 homologue of the HGE agent.

10

	Primer designation	Nucleotide sequence	SEQ ID	
			NO:	Function
	Ehr20 ^a	GCAAGCTTAAGACATGCAAGTC	11	16S rDNA PCR/sequencing
	Ehr241 ^a	CCAGGTATAGATCATCGCC	12	16S rDNA PCR/sequencing
15	HSP961 ^b	GAAGAAATTGCHCAAGTWGC	4	HSP PCR/sequencing
	HSP1754a	TTCTTCAACAGCWGCTCTAG	5	HSP PCR/sequencing
	HSP354	CGYCAGTGGGCTGGTAATGAA	6	HSP PCR/sequencing
	HSP2165	CCATACCWCCCATGCCTC	7	HSP PCR/sequencing
	HSP437	CAAACAACCGTAGCATAAGACTAT	13	HSP sequencing
20	HSP577	AAATCAATTAGAGAAGTTGT	14	HSP sequencing
	HSP611	TAGGACCAGCAGTACAACCAACTGCAT	15	HSP sequencing
	HSP759	TACTCAGAGTGCTTCTCAGTGTAATGA	16	HSP sequencing
	HSP857	CAATATCAGATCCAGCAGCTT	17	HSP sequencing
	HSP1049	AAGACGGTGTTATAACTGTTGAAG	18	HSP sequencing
25	HSP1066	AAKCCTTTRCTTTCTTCAAC	19	HSP sequencing
	HSP1172	TGCTGGTGGAATTTGAAAATC	20	HSP sequencing
	HSP1385	GTGACAGAAGAAAAGACATGCT	21	HSP sequencing
	HSP1411	GATATTGCTGTAATAGTAGGCGCTA	22	HSP sequencing
	HSP1505	CTTTTGTGATTTCGCACGCTCT	23	HSP sequencing
30	HSP1668	TGGCGTTGCTGTACTCAAGGT	24	HSP sequencing
	HSP1719	CATCTTCTACTCTGTCTTTGCGT	25	HSP sequencing
	HSP1750	TCAACAGCAGCTCTAGTTGC	26	HSP sequencing
	HSP1754s	CTAGAGCTGCTGTTGAAGAA	27	HSP sequencing
	HSP1766	ACCAGGTACTACACCTTCCTCAA	28	HSP sequencing

^aRibosomal RNA gene primers reflect the *E. equi* numbering (M73223).

^bHeat shock protein gene primers are based on the numbering for the *E. chaffeensis*

5 HSP60 homologue.

In order to recover the complete open reading frame of the HSP60 homologue from the HGE agent, consensus PCR was performed on HL60 cell cultures co-cultivated with mouse PMNs (see below) isolated from a SCID (C.B-10 17) mouse that was previously infected with patient blood. The patient had clinical and laboratory confirmed HGE infection. Transmission of the HGE agent from the patient to mice was accomplished by intraperitoneal inoculation of 100 µl of blood from the patient into four week-old C.B-17 scid/scid mice (n = 3). Twenty eight days following inoculation, 100 µl of blood was obtained 15 from each mouse by puncture of the peri-orbital sinus and DNA was extracted using the IsoQuick Nucleic Acid Extraction Kit (Orca Research Inc.).

Ehrlichial 16S rDNA was detected in mouse blood by PCR using 16S ehrlichial group-specific primers (e.g., Ehr20 and Ehr241; Table 2). PCR amplification reaction mixes and cycling conditions were identical to those 20 described above for the broad-range HSP60 amplifications with the exception that the cycle number was increased to 50 cycles. Subsequent to PCR, 10 µl of PCR product was electrophoresed in a 2% agarose gel (Seakem GTG, FMC Bioproducts) to assess amplification integrity and the DNA was transferred by Southern blot to a nylon membrane. The presence of ehrlichial 16S rDNA was 25 confirmed by hybridization with an internal probe amplified by PCR primers Ehr552 and Ehr706 (Pancholi et al., *J. Infect. Dis.*, 172, 1007 (1995)). Sequence analysis of the PCR product confirmed that the isolate was HGE (subsequently referred to as the WI-1 HGE isolate).

After confirmation of the transmission of the HGE causative 30 agent, whole blood was obtained from the infected mice by cardiac puncture and the polymorphonuclear leukocytes (PMNs) were isolated by gradient centrifugation. The PMNs were resuspended in Hank's balanced salt solution (BioWhittaker, Inc., Walkersville, MD), and quantitated microscopically in a cell counting chamber. Mouse PMNs were co-cultivated with HL-60 human

promyelocytic leukemia cells (ATCC CCL 240, American Type Culture Collection, Rockville, MD) at a ratio of 1:2 in RPMI 1640 media (BioWhittaker, Inc.) containing 15% fetal calf serum and the infection status of cultured cells was monitored by PCR, as described above for infected mice. DNA was
5 extracted using the IsoQuick kit (Orca Research) from approximately 1.5×10^6 co-cultivated PMN and HL-60 cells 9 days following exposure of the HL-60 cells to infected mouse PMNs.

Broad-range PCR primers HSP354 (SEQ ID NO:6) and HSP2165 (SEQ ID NO:7) (Table 2) were used to perform duplicate amplifications of DNA
10 isolated from HGE agent-infected HL60 cells. Five μ l of the isolated DNA was added to a master mix prepared according to manufacturer's instructions for the Expand High Fidelity PCR System (Boehringer Mannheim Corporation, Indianapolis, IN), with the exceptions that a) 50 pmol of each primer was used in a 100 μ l reaction and b) samples were thermal cycled at 94°C for 4 minutes, then
15 35 cycles at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes, and incubated at 72°C for 10 minutes. The amplified products were electrophoresed in a 1% Seaplaque agarose gel (FMC Bioproducts), the appropriate band was excised, and the DNA products were pooled and purified according to the manufacturer's instructions for the Wizard PCR prep (Promega Corp., Madison,
20 WI).

An approximately 1.8 kb fragment was amplified from the HGE agent-infected cell cultures. Both strands of the product were sequenced with internal primers designed to create overlapping sequences, and a sequence database search was performed on each sequence. The overlapping sequences
25 were then aligned with the Assemblylign Sequence Assembly software package (Oxford Molecular, Campbell, CA) and the contiguous 1.6 kb sequence (SEQ ID NO:1; Figure 1) was used as a query sequence in GenBank to search for related sequences.

Of the related sequences, *C. ruminantium*, *E. chaffeensis*,
30 *Chlamydia pneumoniae*, *Rickettsia tsutsugamushi*, *Bartonella bacilliformis*, and *E. risticii*, were aligned with the ehrlichial sequence using the Pileup algorithm

of the Wisconsin package. The *Escherichia coli* HSP60 gene was included as an outgroup. As previously determined from the human blood-derived sequence, the sequence generated from the HGE agent-infected HL60 cells was most closely related to cognate genes from *E. chaffeensis* and *C. ruminantium* with
5 sequence identity of 75.6% and 75.2%, respectively.

The approximate 1.8 kb DNA segment was then aligned with the *E. chaffeensis* HSP60 sequence by using the GAP algorithm of the Wisconsin package. The alignment indicated that the HGE agent DNA sequence encoding HSP60 comprises a nearly complete open reading frame (ORF), including the
10 ribosomal binding site and start codon, but lacks the terminal 33 bp. The sequence contains 43.3% GC content as compared to *E. chaffeensis* which contains 34.5% GC. Alignment of the predicted HSP60-like amino acid sequences from *E. chaffeensis* and the HGE agent indicated 93.6% amino acid similarity (Figure 2). The codon usage for the HGE agent HSP60-like sequence
15 was similar to that seen in the homologous *E. chaffeensis* sequence for most amino acids, but codon preferences reflected the higher GC content seen in the HGE agent. In addition, the HGE-specific ORF contained an unusual TGG codon for tryptophan which is not seen in *E. chaffeensis* or in the *E. coli* HSP60 gene sequence.

20 Phylogenetic analysis of the alignment generated by the Wisconsin package was accomplished with the Molecular Evolutionary Genetics Analysis (MEGA) 1.01 computer program (Kumar et al., 1.01 ed (1993)). A Jukes-Cantor distance measurement was established and neighbor-joining analysis was performed with 500 bootstrap replicates. Further data analysis was
25 achieved with the Phylogenetic Analysis Using Parsimony (PAUP) 3.1.1 computer program (Swofford, 3.1.1 ed. (1993)). A branch and bound algorithm with 100 bootstrap replicates was used to confirm the branching order observed with the neighbor-joining analysis.

Phylogenetic analyses showed the HGE agent ORF was closely
30 related to those of *C. ruminantium* and *E. chaffeensis* (Figure 3A). However, both neighbor-joining and parsimony analysis indicated that *C. ruminantium* and

E. chaffeensis clustered together in 100% of the bootstrap replicates, to the exclusion of the HGE agent. Rickettsial species *E. risticii* and *Rickettsia tsutsugamushi* clustered with *C. ruminantium*, *E. chaffeensis*, and the HGE agent in 98% and 99% of the bootstrap replicates, respectively, yet did not occupy the same terminal branches.

A similar branching order was provided by analysis of the 16S rRNA gene (Figure 3B). Although it is an arthropod-borne pathogen, with epicellular growth characteristics, *Bartonella bacilliformis* clustered with *E. coli* and segregated from the rickettsia-like agents in 91% of the bootstrap replicates.

Thus, sequence analysis of the HSP60 gene of the HGE agent suggests that the ehrlichial agents associated with HGE are closely related, and that three sequences recovered from patients are similar to, but not identical to, HSP60-like genes described for other bacterial organisms.

Example 2

Preparation of an HGE-Agent Specific Fusion Polypeptide

Useful to Detect Anti-Ehrlichial Antibodies

An 820 bp DNA segment was generated by PCR amplification of HGE-agent infected patient DNA samples with the broad-range primer set HSP961 (SEQ ID NO:4)/HSP1754 (SEQ ID NO:5). The PCR product was electrophoresed in a 2% agarose gel (Seaplaque GTG, FMC Bioproducts), the resulting band was excised, and the DNA was purified by hot phenol extraction and ethanol precipitation (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989)). Single-stranded overhangs were created by digestion with restriction enzymes XbaI and HindIII, resulting in a 540 bp unidirectional insert.

A pGEX-KG plasmid vector (Pharmacia Biotech, Piscataway, NJ) was purified by CsCl gradient (Sambrook et al., *supra*) and digested with XbaI and HindIII. The 540 bp insert was then ligated into the pGEX-KG vector to yield a plasmid that encodes a glutathione S-transferase fusion polypeptide (Glutathione S-Transferase (GST) Gene Fusion System (Pharmacia Biotech Inc.)). Competent *E. coli* DH5 α cells (Gibco-BRL, Gaithersburg, MD) were

used for the initial transformation and maintenance of the insert containing plasmid. Appropriate colonies were chosen and the plasmid was then transformed into *E. coli* BL21 cells for expression of a GST fusion polypeptide. The GST fusion polypeptides were purified by affinity chromatography on
5 glutathione S-transferase 4B beads (Pharmacia Biotech.).

In order to visualize the recombinant GST fusion polypeptide, a 3 µg sample was electrophoresed in a 12% separating/5% stacking SDS-PAGE analytical gel overnight at 80 v and then stained with Coomassie blue. A 45 kDa fusion polypeptide was observed by SDS-PAGE analysis.

10 In order to purify the recombinant GST fusion polypeptide, a preparative 12% separating/5% stacking SDS-PAGE gel was loaded with 30 µg of the GST fusion polypeptide and electrophoresed overnight at 80 v. The GST fusion polypeptide was transferred by Western blot onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford,
15 MA) for 5 hours at 350 mA in a Hoefer Transphor Tank Electrotransfer Unit (Model TE42, Hoefer Scientific, San Francisco, CA) using TOWBIN buffer (192 mM glycine, 25 mM TRIS, pH 8.3, 15% methanol).

Western blots of the recombinant GST fusion polypeptide were employed to detect serum antibody to the HGE agent. Serum antibody directed
20 against the HGE agent in experimentally infected Balb/C mice (infected by syringe inoculation), infected CEH/HeJ mice (infected by tick transmission), and noninfected control mice was qualitatively detected with the Chemiluminescent Blotting Substrate (POD) kit (Boehringer Mannheim Corp., Indianapolis, IN) according to manufacturer's instructions. Briefly, mouse serum was diluted
25 1:250 in 0.5% blocking solution, then applied to the membrane enclosed in a 25 well miniblotted cassette (Immunetics, Inc., Cambridge, MA) and incubated at room temperature for 1 hour on a rocking platform. The blot was washed twice in TRIS buffered sodium chloride with tween 20 (TBST) for 10 minutes and twice in a 0.5% blocking solution for 10 minutes.

30 A secondary reagent of goat anti-mouse Ig polyvalent peroxidase conjugate (Boehringer Mannheim Corp.) diluted 1:4000 in 0.5% blocking

solution was added and the membrane was rocked for 30 minutes at room temperature. Blots were exposed to detection reagents and autoradiography was performed with Kodak X-Omat AR film (Eastman Kodak, Rochester, NY).

Results obtained by Western blot for the detection of antibodies specific for the HSP60-homologue of the HGE agent were confirmed by an indirect fluorescent antibody assay for *E. equi* antigen (Linmed, Biologics, Brea, CA). Sera were screened at a starting dilution of 1:64 in PBS. Fluorescein-conjugated goat anti-mouse IgG was used to detect specific fluorescence of *E. equi*.

Three Balb/C mice were infected with the HGE agent by syringe inoculation of blood from C.B-17 HGE agent infected mice. Infection of the Balb/C mice was confirmed by blood smear and PCR (Figure 4). HGE seroreactivity was confirmed by testing for *E. equi* antibody by IFA. Consistent with the PCR data, a strong antibody response to the 45 kD fusion protein was detected in all three infected Balb/C mice 28 days after inoculation (Figure 5A). In contrast, no reactivity was observed in mice infected with *B. burgdorferi* strain N40 or in mice inoculated with blood from uninfected C.B-17 mice.

To determine whether the immunoreactivity in infected mice was specific for the ehrlichial portion of the fusion protein, the sera was screened against a GST polypeptide (Figure 5B). None of the sera reacted with the GST polypeptide under conditions in which the cleaved GST polypeptide was detected by a GST-specific antibody. Thus, the HGE-specific portion of the fusion polypeptide is immunogenic.

Figure 5C shows the results from C3H/HeJ mice infected by tick transmission with an east coast strain (NCH-1) of the HGE agent or with *B. burgdorferi* strain N40. The east coast strain of the HGE agent was isolated from a patient from Nantucket Island, MA. Deer ticks experimentally infected with the NCH-1 strain of the HGE agent were allowed to feed upon nine C3H/HeJ mice as described by Telford et al., Proc. Natl. Acad. Sci. USA, **93**, 6209 (1996). The mice were then followed for up to 19 months by direct smear evaluation and *E. equi* IFA.

Antibody responses to affinity purified ehrlichial HGE agent HSP-60 antigen could also be detected for six or more months after infection. However, the level of reactivity on Western blot correlated qualitatively rather than quantitatively with the IFA titers (Figure 5C). One mouse, infected for six months, was weakly seroreactive with the fusion polypeptide, yet was IFA negative at a 1:64 screening titer (Figure 5C, lane 7). This discordance was not observed with any of the other mice, infected or uninfected, and may be due to a cut off titer (1:64) that is too high.

Two C3H/HeJ mice which were fed upon by infected *Ixodes* sp. ticks were negative by blood smear evaluation. These mice also had no significant antibody response to *E. equi* antigens, as determined by the *E. equi* IFA assay, or to the HGE-specific HSP-60 fusion polypeptide (Figure 5C, lanes 3 and 11). Negative controls included sera from three mice that were fed upon by uninfected ticks and two mice infected with the N40 strain of *B. burgdorferi* by tick inoculation. None of the sera from these negative control animals showed reactivity to the 45 kD fusion polypeptide. However, one serum from a *Borrelia* infected animal did show background reactivity which may be due to the presence of *E. coli* proteins which co-purified with the fusion polypeptide (lane 16).

Thus, the HSP60-like polypeptide and other immunogenic polypeptides from the agent which is associated with HGE agent can be used as immunodiagnostic reagents for immunodiagnostic testing for infections with this agent. Thus, these reagents may be useful to detect HGE specific infections in humans and other mammals, such as deer.

Moreover, this reagent may also be used to detect monocytic ehrlichiosis due to *E. chaffeensis* infection, in light of the prevalence of *E. chaffeensis* infection and the relatedness of the agent which is associated with HGE to *E. chaffeensis*. Therefore, immunoreactive polypeptides of the invention may comprise genus-specific epitopes, species-specific epitopes, or both.

The invention is not limited to the exact details shown and described, for it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention defined by the claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Mayo Foundation for Medical Education and Research

(ii) TITLE OF THE INVENTION: METHODS TO DETECT GRANULOCYTIC
EHRlichiosis

(iii) NUMBER OF SEQUENCES: 28

(iv) CORRESPONDENCE ADDRESS:

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- (B) STREET: P.O. Box 2938
- (C) CITY: Minneapolis
- (D) STATE: MN
- (E) COUNTRY: USA
- (F) ZIP: 55402

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: 08/828,199
- (B) FILING DATE: 21 MAR 1997
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Embretson, Janet E
- (B) REGISTRATION NUMBER: 39,665
- (C) REFERENCE/DOCKET NUMBER: 150.178WO1

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 612-373-6959
- (B) TELEFAX: 612-339-3061
- (C) TELEX: N/A

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1777 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

CAGTGGGCTG GTAATGAAAT AGAGTTCGAC GGTAAGAAGT TCATAGTGAT GAAGGAGAGT    60
GACATAATAG CTAAGGAAGC ATAGTCTTAT GCTACGGTTG TTTGTTCTAT TGGTCTAAAG    120
TTTAGGAGGT TCAAAAATGT CAAATACGGT AGTCACGGGT GAGGTATTAG ATAAGTCTAT    180
TAGGGAGGTA GTACGCATCC TAGAAGATGC AGTTGGTTGT ACTGCTGGTC CTAAAGGCCCT    240
CACTGTAGCG ATTAGTAAGC CTTATGGGTC GCCAGAGATC ACAAAGGATG GCTACAAGGT    300
AATGAAGAGT ATAAAGCCTG AAGAACCCTT GGCTGCCGCT ATAGCGAGCA TCATTACTCA    360
GAGTGCTTCT CAGTGTAATG ATAAGGTGGG AGATGGAAGT ACTACATGCT CCATACTAAC    420
GGCAAAAGTG ATTGAAGAAG TCTCAAAAGC GAAAGCTGCT GGATCTGATA TTGTTAGCAT    480
AAAGAATGGT ATTCTCAAGG CTAAGGAAGC GGTTCTTACA GCGCTTATGT CTATGAGACG    540
TGAAGTAGAA GAAGACGAAA TTGCACAAGT TGCAACATTG TCTGCGAATG GAGACAAGAA    600
CATAGGAAGT AAGATTGCAC AGTGTGTTAA AGAAGTCGGT AAAGACGGTG TTATACTGT    660
TGAAGAAAAGC AAAGGCTTCA AGGATCTAGA AGTTGAAAAG ACTGATGGTA TGCAGTTTGA    720
TCGCGGATAT CTTTCGCCTT ACTTTGTTAC AAATGCTGAA AAAATGCTGG TGAATTGGA    780
AAATCCATAC ATATTCCTTA CTGAAAAGAA GATTAATCTT GTACAAAGCA TTCTACCAAT    840
CTTAGAAAAC GTTGACACGGT CTGGAAGACC ATTGCTCATC ATAGCTGAAG ACGTTGAAGG    900
TGAAGCTCTG AGCACGCTTG TACTCAATAA GCTCCGTGGT GGCCTTCAAG TTGCTGCTGT    960
AAAGGCGCCT GGTTCGCTG ACAGAAGAAA AGACATGCTT GGCGATATG CTGTAATAGT   1020
AGGCGCTAAG TATGTAGTAA ATGACGAGCT TGCTGTAAAG ATGGAAGACA TCGCTCTAAG   1080
CGATCTTGGT ACTGCTAAGA GCGTGCGAAT CACAAAAGAC GCAACTACTA TTATAGGTAG   1140
TGTTGATAGC AGTTCGAAA GCATAGCTAG CAGGACTAAT CAAATCAAAG CTCAGATAGA   1200
AAATTCAGT TCTGATTATG ACAAGGAAAA GCTTAGAGAA CGTTTAGCGA AGCTTCCCGG   1260
TGGCGTTGCT TTAACAAGG TTGGTGGATC CAGCGAAGTT GAGGTGAAGG AACGCAAAGA   1320
CAGAGTAGAA GATGCTTTAC ATGCTACTAG AGCTGCTGTT GAGGAAGGTG TAGTACCTGG   1380
TGGTGGGGCT GCATTGCTTT ATGCGCTTTC GTCTCTAGAC GGTCTAAAAG GCAAGAATGA   1440
CGACGAACAA TGGGGTATAG ACATTATACG TCGCGCTGCT TGTGCTCCAA TCAAAAGAAT   1500
CATCAAGAAT TCTGGTCTG AAGAAGCACC ATGCGTAATT CAACACTTGT TGAAGCAAAA   1560
CGACAAGGAA CTTATCTACA ATGTGGATAC TATGAAGTAC GCGAATGCTT TTACATCTGG   1620
AGTTATGGAT CCTCTCAAAG TAGTACGTAT CGCGTTTGAT TTAGCTGTAT CACTCGCTGC   1680
AGTATTCATG ACTTTGAATG CAGTGGTTGT TGATGTTCCCT AGTAAGAACG ACGCTGCTGG   1740
TGCTGGCGCT GGTGGTATGG GAGGCATGGG TGGTATG                                1777

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 541 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Ser Asn Thr Val Val Thr Gly Glu Val Leu Asp Lys Ser Ile Arg
 1             5             10             15
Glu Val Val Arg Ile Leu Glu Asp Ala Val Gly Cys Thr Ala Gly Pro
          20          25          30
Lys Gly Leu Thr Val Ala Ile Ser Lys Pro Tyr Gly Ser Pro Glu Ile

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35	40	45
Thr Lys Asp Gly Tyr Lys Val Met Lys Ser Ile Lys Pro Glu Glu Pro		
50	55	60
Leu Ala Ala Ala Ile Ala Ser Ile Ile Thr Gln Ser Ala Ser Gln Cys		
65	70	75
Asn Asp Lys Val Gly Asp Gly Thr Thr Thr Cys Ser Ile Leu Thr Ala		
85	90	95
Lys Val Ile Glu Glu Val Ser Lys Ala Lys Ala Ala Gly Ser Asp Ile		
100	105	110
Val Ser Ile Lys Asn Gly Ile Leu Lys Ala Lys Glu Ala Val Leu Thr		
115	120	125
Ala Leu Met Ser Met Arg Arg Glu Val Glu Glu Asp Glu Ile Ala Gln		
130	135	140
Val Ala Thr Leu Ser Ala Asn Gly Asp Lys Asn Ile Gly Ser Lys Ile		
145	150	155
Ala Gln Cys Val Lys Glu Val Gly Lys Asp Gly Val Ile Thr Val Glu		
165	170	175
Glu Ser Lys Gly Phe Lys Asp Leu Glu Val Glu Lys Thr Asp Gly Met		
180	185	190
Gln Phe Asp Arg Gly Tyr Leu Ser Pro Tyr Phe Val Thr Asn Ala Glu		
195	200	205
Lys Met Leu Val Glu Phe Glu Asn Pro Tyr Ile Phe Leu Thr Glu Lys		
210	215	220
Lys Ile Asn Leu Val Gln Ser Ile Leu Pro Ile Leu Glu Asn Val Ala		
225	230	235
Arg Ser Gly Arg Pro Leu Leu Ile Ile Ala Glu Asp Val Glu Gly Glu		
245	250	255
Ala Leu Ser Thr Leu Val Leu Asn Lys Leu Arg Gly Gly Leu Gln Val		
260	265	270
Ala Ala Val Lys Ala Pro Gly Phe Gly Asp Arg Arg Lys Asp Met Leu		
275	280	285
Gly Asp Ile Ala Val Ile Val Gly Ala Lys Tyr Val Val Asn Asp Glu		
290	295	300
Leu Ala Val Lys Met Glu Asp Ile Ala Leu Ser Asp Leu Gly Thr Ala		
305	310	315
Lys Ser Val Arg Ile Thr Lys Asp Ala Thr Thr Ile Ile Gly Ser Val		
325	330	335
Asp Ser Ser Ser Glu Ser Ile Ala Ser Arg Thr Asn Gln Ile Lys Ala		
340	345	350
Gln Ile Glu Asn Ser Ser Ser Asp Tyr Asp Lys Glu Lys Leu Arg Glu		
355	360	365
Arg Leu Ala Lys Leu Ser Gly Gly Val Ala Val Leu Lys Val Gly Gly		
370	375	380
Ser Ser Glu Val Glu Val Lys Glu Arg Lys Asp Arg Val Glu Asp Ala		
385	390	395
Leu His Ala Thr Arg Ala Ala Val Glu Glu Gly Val Val Pro Gly Gly		
405	410	415
Gly Ala Ala Leu Leu Tyr Ala Leu Ser Ser Leu Asp Gly Leu Lys Gly		
420	425	430
Lys Asn Asp Asp Glu Gln Trp Gly Ile Asp Ile Ile Arg Arg Ala Ala		
435	440	445
Cys Ala Pro Ile Lys Arg Ile Ile Lys Asn Ser Gly Ser Glu Glu Ala		
450	455	460
Pro Cys Val Ile Gln His Leu Leu Lys Gln Asn Asp Lys Glu Leu Ile		
465	470	475
		480

40

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Tyr Asn Val Asp Thr Met Asn Tyr Ala Asn Ala Phe Thr Ser Gly Val
      485                      490                      495
Met Asp Pro Leu Lys Val Val Arg Ile Ala Phe Asp Leu Ala Val Ser
      500                      505                      510
Leu Ala Ala Val Phe Met Thr Leu Asn Ala Val Val Val Asp Val Pro
      515                      520                      525
Ser Lys Asn Asp Ala Ala Gly Ala Gly Gly Met
      530                      535                      540

```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 549 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Met Ala Asn Val Val Val Thr Gly Glu Gln Leu Asp Lys Ser Ile Arg
 1      5      10      15
Glu Val Val Arg Ile Leu Glu Asp Ala Val Gly Cys Thr Ala Gly Pro
      20      25      30
Lys Gly Leu Thr Val Ala Ile Gly Lys Ser Tyr Gly Ala Pro Glu Val
      35      40      45
Thr Lys Asp Gly Tyr Lys Val Ile Lys Ser Ile Lys Pro Glu Asp Pro
      50      55      60
Leu Ala Leu Ala Ile Ala Asn Ile Ile Thr Gln Ser Ala Ser Gln Cys
65      70      75      80
Asn Asp Lys Val Gly Asp Gly Thr Thr Thr Cys Ser Ile Leu Thr Ala
      85      90      95
Lys Val Ile Glu Glu Val Ser Lys Ala Lys Ala Ala Gly Ala Asp Ile
      100     105     110
Val Cys Ile Lys Glu Gly Val Leu Lys Ala Lys Glu Ala Val Leu Glu
      115     120     125
Ala Leu Met Ser Met Lys Arg Glu Val Leu Ser Glu Glu Glu Ile Ala
      130     135     140
Gln Val Ala Thr Ile Ser Ala Asn Gly Asp Lys Asn Ile Gly Ser Lys
      145     150     155     160
Ile Ala Gln Cys Val Gln Glu Val Gly Lys Asp Gly Val Ile Thr Val
      165     170     175
Glu Glu Ser Lys Gly Phe Lys Glu Leu Asp Val Glu Lys Thr Asp Gly
      180     185     190
Met Gln Phe Asp Arg Gly Tyr Leu Ser Pro Tyr Phe Val Thr Asn Ser
      195     200     205
Glu Lys Met Leu Val Glu Phe Glu Asn Pro Tyr Ile Leu Leu Thr Glu
      210     215     220
Lys Lys Leu Asn Ile Ile Gln Pro Ile Leu Pro Ile Leu Glu Asn Val
      225     230     235     240
Ala Arg Ser Gly Arg Pro Leu Leu Ile Ile Ala Glu Asp Val Glu Gly
      245     250     255
Glu Ala Leu Ser Thr Leu Val Leu Asn Lys Leu Arg Gly Gly Leu His

```

41

	260		265		270										
Val	Ala	Ala	Val	Lys	Ala	Pro	Gly	Phe	Gly	Asp	Arg	Arg	Lys	Asp	Met
	275		280		285										
Leu	Gly	Asp	Ile	Ala	Ile	Leu	Thr	Gly	Ala	Lys	His	Val	Ile	Ser	Asp
	290		295		300										
Asp	Leu	Ala	Ile	Lys	Met	Glu	Asp	Leu	Thr	Leu	Ala	Glu	Leu	Gly	Thr
	305		310		315										320
Ala	Lys	Asn	Ile	Arg	Ile	Thr	Lys	Asp	Thr	Thr	Thr	Ile	Ile	Gly	Ser
		325						330						335	
Val	Asp	Asn	Ser	Ser	Ala	Asn	Val	Gln	Ser	Arg	Ile	Asn	Gln	Ile	Lys
	340						345					350			
Met	Gln	Ile	Glu	Ala	Ser	Thr	Ser	Asp	Tyr	Asp	Lys	Glu	Lys	Leu	Arg
	355						360					365			
Glu	Arg	Leu	Ala	Lys	Leu	Ser	Gly	Gly	Val	Ala	Val	Leu	Lys	Val	Gly
	370					375					380				
Gly	Ser	Ser	Glu	Val	Glu	Val	Lys	Glu	Arg	Lys	Asp	Arg	Val	Glu	Asp
	385				390					395					400
Ala	Leu	His	Ala	Thr	Arg	Ala	Ala	Val	Glu	Glu	Gly	Val	Val	Pro	Gly
			405						410					415	
Gly	Gly	Ala	Ala	Leu	Leu	Tyr	Thr	Leu	Ser	Val	Leu	Glu	Asn	Leu	Lys
		420					425						430		
Ser	Lys	Asn	Asp	Asp	Glu	Gln	Leu	Gly	Ile	Asn	Ile	Val	Lys	Arg	Ala
	435						440					445			
Leu	Gln	Ala	Pro	Ile	Lys	Arg	Ile	Ile	Lys	Asn	Ser	Gly	Ser	Glu	Asn
	450					455					460				
Ala	Pro	Cys	Val	Ile	Ala	His	Leu	Leu	Lys	Gln	Asn	Asp	Lys	Glu	Leu
	465				470					475					480
Ile	Phe	Asn	Val	Asp	Thr	Met	Asn	Phe	Ala	Asn	Ala	Phe	Thr	Ser	Gly
			485					490						495	
Val	Ile	Asp	Pro	Leu	Lys	Val	Val	Arg	Ile	Ala	Phe	Asp	Phe	Ala	Val
		500					505					510			
Ser	Leu	Ala	Ala	Val	Phe	Met	Thr	Leu	Asn	Ala	Ile	Val	Val	Asp	Val
	515						520					525			
Pro	Ser	Lys	Asp	Asp	Ala	Asn	Ala	Gly	Ala	Gly	Gly	Met	Gly	Gly	Met
	530					535					540				
Gly	Gly	Met	Gly	Gly											
	545														

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAAGAAATTG CHCAAGTWGC

42

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: YES

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTCTTCAACA GCWGCTCTAG

20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGYCAGTGGG CTGGTAATGA A

21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: YES

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCATACCWCC CATGCCTC

18

(2) INFORMATION FOR SEQ ID NO:8:

43

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

YGGATAYCTT TCKCCTTAYT TT

22

- (2) INFORMATION FOR SEQ ID NO:9:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: oligonucleotide
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTISENSE: NO
 - (v) FRAGMENT TYPE:
 - (vi) ORIGINAL SOURCE:
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTTGGTACTG CTAAGAGCGT G

21

- (2) INFORMATION FOR SEQ ID NO:10:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: oligonucleotide
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTISENSE: NO
 - (v) FRAGMENT TYPE:
 - (vi) ORIGINAL SOURCE:
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCTTCCTCAA CAGCAGCTCT A

21

- (2) INFORMATION FOR SEQ ID NO:11:
 - (i) SEQUENCE CHARACTERISTICS:

44

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCAAGCTTAA GACATGCAAG TC

22

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCAGGTATAG ATCATCGCC

19

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CAAACAACCG TAGCATAAGA CTAT

24

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs

45

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAATCAATTA GAGAAGTTGT

20

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TAGGACCAGC AGTACAACCA ACTGCAT

27

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TACTCAGAGT GCTTCTCAGT GTAATGA

27

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid

46

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CAATATCAGA TCCAGCAGCT T

21

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAGACGGTGT TATAACTGTT GAAG

24

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAKCCTTTRC TTTCTTCAAC

20

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TGCTGGTGGA ATTTGAAAAT C

21

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTGACAGAAG AAAAGACATG CT

22

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATATTGCTG TAATAGTAGG CGCTA

25

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

48

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTTTTGTGAT TCGCACGCTC T

21

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TGGCGTTGCT GTACTCAAGG T

21

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CATCTTCTAC TCTGTCTTTG CGT

23

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: oligonucleotide

- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TCAACAGCAG CTCTAGTTGC

20

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTAGAGCTGC TGTGAAGAA

20

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: YES

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

ACCAGGTACT ACACCTTCCT CAA

23

WHAT IS CLAIMED IS:

1. An isolated and purified DNA molecule comprising a preselected DNA sequence which encodes an immunogenic subunit of a polypeptide or a biologically active variant thereof, wherein the expression of the polypeptide in a host is associated with granulocytic ehrlichiosis in a mammal.
2. The DNA molecule of claim 1 wherein the preselected DNA sequence is a contiguous fragment of SEQ ID NO:1.
3. The DNA molecule of claim 1 wherein the preselected DNA sequence encodes an immunogenic subunit of SEQ ID NO:2.
4. An isolated and purified DNA molecule which is complementary to the DNA molecule of claim 2.
5. An expression cassette comprising: a preselected DNA sequence which is operably linked to a promoter functional in a host cell, which DNA sequence encodes an immunogenic polypeptide, a biologically active subunit or a biologically active variant thereof, which is associated with granulocytic ehrlichiosis in a mammal.
6. The expression cassette of claim 5 wherein the preselected DNA sequence comprises SEQ ID NO:1.
7. The expression cassette of claim 5 wherein the preselected DNA sequence encodes a polypeptide having an amino acid sequence comprising SEQ ID NO:2.
8. The expression cassette of claim 5 wherein the preselected DNA

sequence encodes a fusion polypeptide comprising said immunogenic polypeptide.

9. An isolated and purified immunogenic polypeptide, a biologically active subunit or variant thereof, which is associated with granulocytic ehrlichiosis in a mammal.
10. The isolated and purified polypeptide of claim 9 which comprises the amino acid sequence comprising SEQ ID NO:2.
11. A fusion polypeptide comprising the isolated and purified polypeptide of claim 9.
12. An immunogenic composition comprising a polypeptide, a variant or subunit thereof, the presence of which is associated with granulocytic ehrlichiosis in a mammal, in combination with a pharmaceutically acceptable carrier, wherein the administration of the immunogenic composition to a mammal induces the production of antibodies to the polypeptide.
13. The immunogenic composition or vaccine of claim 12 wherein the polypeptide has an amino acid sequence comprising SEQ ID NO:2.
14. A method of using a nucleic acid molecule encoding an immunogenic polypeptide, the presence of which is associated with granulocytic ehrlichiosis in a mammal, said method comprising expressing the nucleic acid molecule in a cultured host cell stably transformed with a chimeric vector comprising said nucleic acid molecule operably linked to control sequences recognized by the host cell, and recovering the polypeptide from the host cell.

15. The method of claim 14 wherein the nucleic acid molecule comprises SEQ ID NO:1.
16. The method of claim 14 wherein the polypeptide has an amino acid
5 sequence comprising SEQ ID NO:2.
17. A method for detecting or determining the presence antibodies which are specific for an infectious agent that is associated with granulocytic ehrlichiosis, in a mammalian physiological sample, which method
10 comprises:
- (a) contacting an amount of purified immunogenic polypeptide encoded by a nucleic acid of the infectious agent with the mammalian physiological sample which comprises antibodies suspected of specifically reacting with the infectious agent, for a
15 sufficient time to form binary complexes between at least a portion of the antibodies and a portion of the purified polypeptide; and
- (b) detecting or determining the presence or amount of said binary complexes.
- 20
18. A method for detecting granulocytic ehrlichiosis in a mammal, comprising:
- (a) contacting an amount of purified immunogenic polypeptide encoded by a nucleic acid of the infectious agent with the
25 mammalian physiological sample which comprises antibodies suspected of specifically reacting with an infectious agent associated with granulocytic ehrlichiosis, for a sufficient time to form binary complexes between at least a portion of the antibodies and a portion of the purified polypeptide; and
- 30 (b) detecting or determining the presence or amount of the binary complexes, wherein the presence or amount of said complexes is

indicative of a mammal at risk of, or afflicted with, granulocytic ehrlichiosis.

19. The method of claim 17 or 18 wherein the purified polypeptide has an amino acid sequence comprising SEQ ID NO:2.
20. A diagnostic kit for detecting or determining the presence of antibodies that specifically react with an infectious agent which is associated with granulocytic ehrlichiosis, which comprises packaging, containing, separately packaged:
- (a) a solid phase capable of having attached thereto a polypeptide; and
 - (b) a known amount of a purified polypeptide which specifically reacts with antibodies specific for the agent.
21. The kit of claim 20 wherein the polypeptide has an amino acid sequence comprising SEQ ID NO:2.
22. A diagnostic method, comprising:
- (a) contacting an amount of DNA obtained from a physiological sample which comprises cells from a mammal at risk of, or afflicted with, granulocytic ehrlichiosis, with an amount of at least two oligonucleotides under conditions effective to amplify the DNA by a polymerase chain reaction so as to yield an amount of amplified DNA, wherein at least one oligonucleotide is specific for DNA encoding a polypeptide of an infectious agent which is associated with granulocytic ehrlichiosis; and wherein
 - (b) detecting or determining the presence of the amplified DNA, wherein the presence of the amplified DNA is indicative of a mammal afflicted with, or at risk of, granulocytic ehrlichiosis.

23. A method for detecting DNA encoding an immunogenic polypeptide associated with granulocytic ehrlichiosis in a mammal, comprising:
- 5 (a) contacting an amount of DNA obtained from a mammalian physiological sample which comprises cells suspected of containing DNA encoding the immunogenic polypeptide, with an amount of at least two oligonucleotides under conditions effective to amplify the DNA by a polymerase chain reaction so as to yield an amount of amplified DNA, wherein at least one oligonucleotide is specific for the DNA encoding the immunogenic polypeptide; and
- 10 (b) detecting or determining the presence of the amplified DNA.
24. The method of claim 22 or 23 wherein the physiological sample is a fluid.
- 15 25. The method of claim 24 wherein the fluid is whole blood.
26. The method of claim 24 wherein the fluid is blood plasma.
- 20 27. The method of claim 22 or 23 wherein the amplified DNA is subjected to agarose gel electrophoresis prior to detection.
28. The method of claim 22 or 23 wherein one oligonucleotide comprises SEQ ID NO:4.
- 25 29. The method of claim 22 or 23 wherein one oligonucleotide comprises SEQ ID NO:5.
- 30 30. The method of claim 22 or 23 wherein one oligonucleotide comprises SEQ ID NO:6.

31. The method of claim 22 or 23 wherein one oligonucleotide comprises SEQ ID NO:7.
32. A diagnostic kit for detecting the presence of DNA associated with granulocytic ehrlichiosis in a sample, which comprises packaging containing (a) a known amount of a first oligonucleotide, wherein the first oligonucleotide consists of at least about 7-50 nucleotides, and wherein the oligonucleotide has at least about 80% identity to SEQ ID NO:1, and (b) a known amount of a second oligonucleotide, wherein the second oligonucleotide consists of at least about 7-50 nucleotides, and wherein the oligonucleotide has at least about 80% identity to a nucleotide sequence which is complementary to SEQ ID NO:1.
33. An oligonucleotide which consists of at least about 7-50 nucleotides, and which has at least about 80% identity to, or is complementary to, a nucleotide sequence comprising SEQ ID NO:1.
34. An oligonucleotide which consists of at least about 7-50 nucleotides, and which has at least about 80% identity to a nucleotide sequence comprising SEQ ID NO:8.
35. An oligonucleotide which consists of at least about 7-50 nucleotides, and which has at least about 80% identity to a nucleotide sequence comprising SEQ ID NO:9.
36. An oligonucleotide which consists of at least about 7-50 nucleotides, and which has at least about 80% identity to a nucleotide sequence comprising SEQ ID NO:10.

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1 CAGTGGGCTG GTAATGAAAT AGAGTTCGAC GGTAAGAAGT TCATAGTGAT
51 GAAGGAGAGT GACATAATAG CTAAGGAAGC ATAGTCTTAT GCTACGGTTG
101 TTTGTTCTAT TGGTCTAAAG TTTAGGAGGT TCAAAAATGT CAAATACGGT
151 AGTCACGGGT GAGGTATTAG ATAAGTCTAT TAGGGAGGTA GTACGCATCC
201 TAGAAGATGC AGTTGGTTGT ACTGCTGGTC CTAAAGGCCT CACTGTAGGG
251 ATTAGTAAGC CTTATGGGTC GCCAGAGATC ACAAAGGATG GCTACAAGGT
301 AATGAAGAGT ATAAAGCCTG AAGAACCACT GGCTGCCGCT ATAGCGAGCA
351 TCATTACTCA GAGTGCTTCT CAGTGTAATG ATAAGGTGGG AGATGGAAC
401 ACTACATGCT CCATACTAAC GGCAAAAGTG ATTGAAGAAG TCTCAAAAGC
451 GAAAGCTGCT GGATCTGATA TTGTTAGCAT AAAGAATGGT ATTCTCAAGG
501 CTAAGGAAGC GGTTCTTACA GCGCTTATGT CTATGAGACG TGAAGTAGAA
551 GAAGACGAAA TTGCACAAGT TGCAACATTG TCTGCGAATG GAGACAAGAA
601 CATAGGAAGT AAGATTGCAC AGTGTGTTAA AGAAGTCGGT AAAGACGGTG
651 TTATAACTGT TGAAGAAAGC AAAGGCTTCA AGGATCTAGA AGTTGAAAAG
701 ACTGATGGTA TGCAGTTTGA TCGCGGATAT CTTTCGCCTT ACTTTGTTAC
751 AAATGCTGAA AAAATGCTGG TGGAATTTGA AAATCCATAC ATATTCCTTA
801 CTGAAAAGAA GATTAATCTT GTACAAAGCA TTCTACCAAT CTTAGAAAAC
851 GTTGACCGGT CTGGAAGACC ATTGCTCATC ATAGCTGAAG ACGTTGAAGG
901 TGAAGCTCTG AGCACGCTTG TACTCAATAA GCTCCGTGGT GGCCTTCAAG
951 TTGCTGCTGT AAAGGCGCCT GGTTTCGGTG ACAGAAGAAA AGACATGCTT
1001 GGCGATATTG CTGTAATAGT AGGCGCTAAG TATGTAGTAA ATGACGAGCT
1051 TGCTGTAAAG ATGGAAGACA TCGCTCTAAG CGATCTTGGT ACTGCTAAGA

FIG. 1
SUBSTITUTE SHEET (RULE 26)

1101 GCGTGCGAAT CACAAAAGAC GCAACTACTA TTATAGGTAG TGTTGATAGC
1151 AGTTCTGAAA GCATAGCTAG CAGGACTAAT CAAATCAAAG CTCAGATAGA
1201 AAATTCTAGT TCTGATTATG ACAAGGAAAA GCTTAGAGAA CGTTTAGCGA
1251 AGCTTTCCGG TGGCGTTGCT GTACTCAAGG TTGGTGGATC CAGCGAAGTT
1301 GAGGTGAAGG AACGCAAAGA CAGAGTAGAA GATGCTTTAC ATGCTACTAG
1351 AGCTGCTGTT GAGGAAGGTG TAGTACCTGG TGGTGGGGCT GCATTGCTTT
1401 ATGCGCTTTC GTCTCTAGAC GGTCTAAAAG GCAAGAATGA CGACGAACAA
1451 TGGGGTATAG ACATTATACG TCGCGCTGCT TGTGCTCCAA TCAAAGAAT
1501 CATCAAGAAT TCTGGTTCTG AAGAAGCACC ATGCGTAATT CAACACTTGT
1551 TGAAGCAAAA CGACAAGGAA CTTATCTACA ATGTGGATAC TATGAACTAC
1601 GCGAATGCTT TTACATCTGG AGTTATGGAT CCTCTCAAAG TAGTACGTAT
1651 CGCGTTTGAT TTAGCTGTAT CACTCGCTGC AGTATTCATG ACTTTGAATG
1701 CAGTGGTTGT TGATGTTCTT AGTAAGAACG ACGCTGCTGG TGCTGGCGCT
1751 GGTGGTATGG GAGGCATGGG TGGTATG

FIG. 1 (Continued)

HGE agent	1	MSNTVVTGEVLDKSIREVVRILEDAGGCTAGPKGLTVAISKPYGSPEITK	50
		. . : . .:	
E. chaffeensis	1	MANVVVTGEQLDKSIREVVRILEDAGGCTAGPKGLTVAIGKSYGAPEVTK	50
		
	51	DGYKVMKSIIKPEEPLAAAIASIIITQSASQCNDKVGDGTTTCISILTAKVIE	100
		:	
	51	DGYKVIKSIKPEDPLALAIANIIITQSASQCNDKVGDGTTTCISILTAKVIE	100
		
	101	EVS KAKAAGSDIVSIKN GILKAKEAVLTALMSMRREV.EEDEIAQVATLS	149
		.: : : : .: : : : .: : : :	
	101	EVS KAKAAGADIVCIKEGV LKAKEAVLEALMSMKREVLSEEEIAQVATIS	150
		
	150	ANGDKNIGSKIAQC VKEVGKDGVITVEESKGFKDLEVEKT DGMQFDRGYL	199
		.: : : :	
	151	ANGDKNIGSKIAQC VQEVGKDGVITVEESKGFKELDV EKT DGMQFDRGYL	200
		
	200	SPYFVTNAEKMLVEFENPYIFLTEKKINLVQSILPILENVARSGRPLLII	249
		.: : : : .: :~::~ .	
	201	SPYFVTNSEKMLVEFENPYILLTEKKLNIIQPILPILENVARSGRPLLII	250
		
	250	AEDVEGEALSTLV LNKL RGG LQVA AVKAPGF GDRRK DMLGDI AIVIGAKY	299
		.: : : : :.: .	
	251	AEDVEGEALSTLV LNKL RGG LHVA AVKAPGF GDRRK DMLGDI AILTGA KH	300
		
	300	VVNDELAVKMEDIA LSDLGTAKSVRITKDATTIIGSVDSSES IASRTNQ	349
		.: : : : .: : : : .: : : : .: : : : .:~::~ .	
	301	VISDDLAIKMEDLTLA ELGTAKNIRITKDTTTTIIGSVDNSS ANVQSRINQ	350
		
	350	IKAQIENS SSSDYD KEKLRRERLAKLSGGVAVLKVGGS SEVEVKERKDRVED	399
		. .: : : :	
	351	IKMQIEASTSDYD KEKLRRERLAKLSGGVAVLKVGGS SEVEVKERKDRVED	400
		
	400	ALHATRAAVEEGVVP GGGAALLYALSSLDGLKGKNDDEQWGIDIIRRAAC	449
		.: :~::~ :~::~ :~::~	
	401	ALHATRAAVEEGVVP GGGAALLYT LSVLENLKS KNDEQLGINIVKRALQ	450
		
	450	APIKRI IKNSGSEE APCV IQHL LKQNDKELIYNVD TMNYANAFTSGVMDP	499
		.: : : : :	
	451	APIKRI IKNSGSEN APCV IAHL LKQNDKELIFNVDT MNFANAFTSGVIDP	500
		
	500	LKVVRIFA FDLAVSLAAVFMTLNAVVD VP SKNDAAGAGAGGM.....	541
		: : : : .: : : :	
	501	LKVVRIFA FDFAVSLAAVFMTLNAIVVD VP SKDD.ANAGAGGMGGMGGMGG	549

FIG. 2

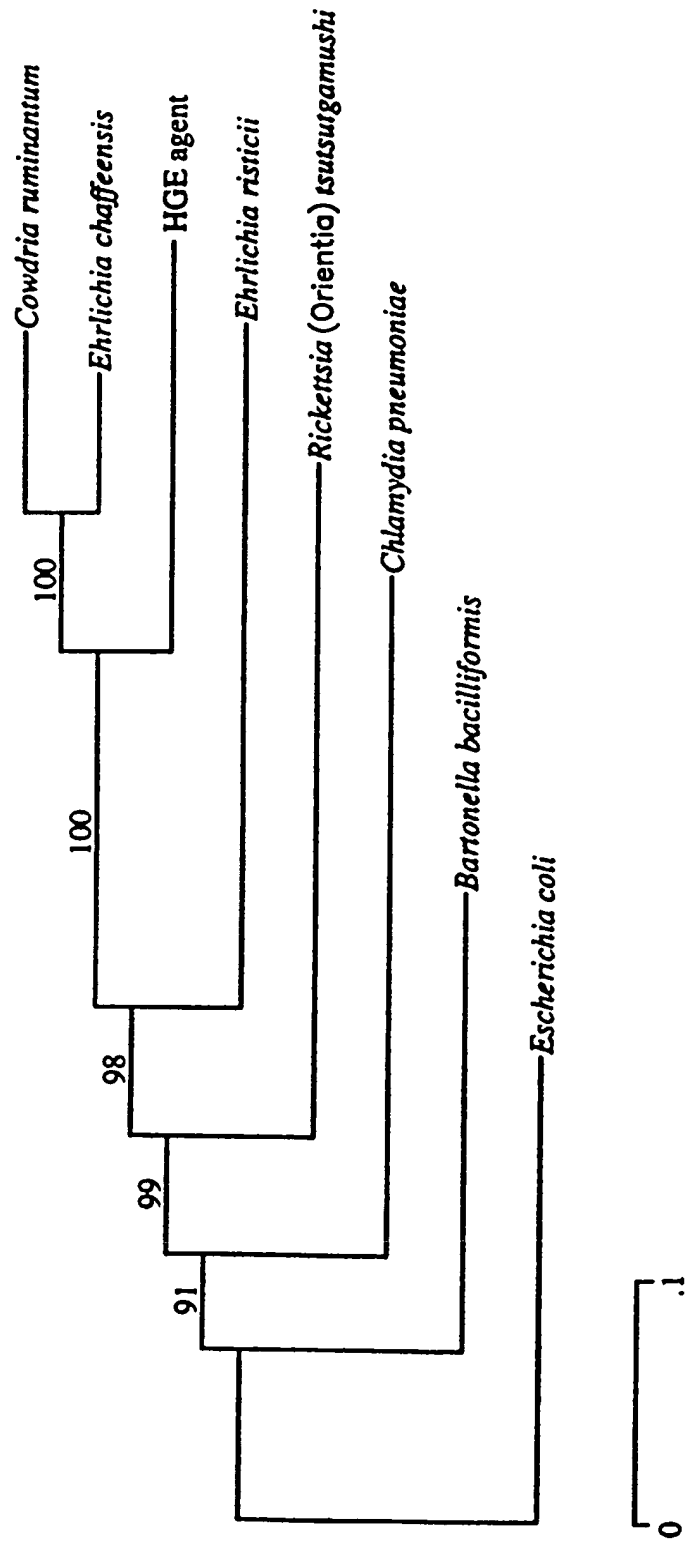


FIG. 3A

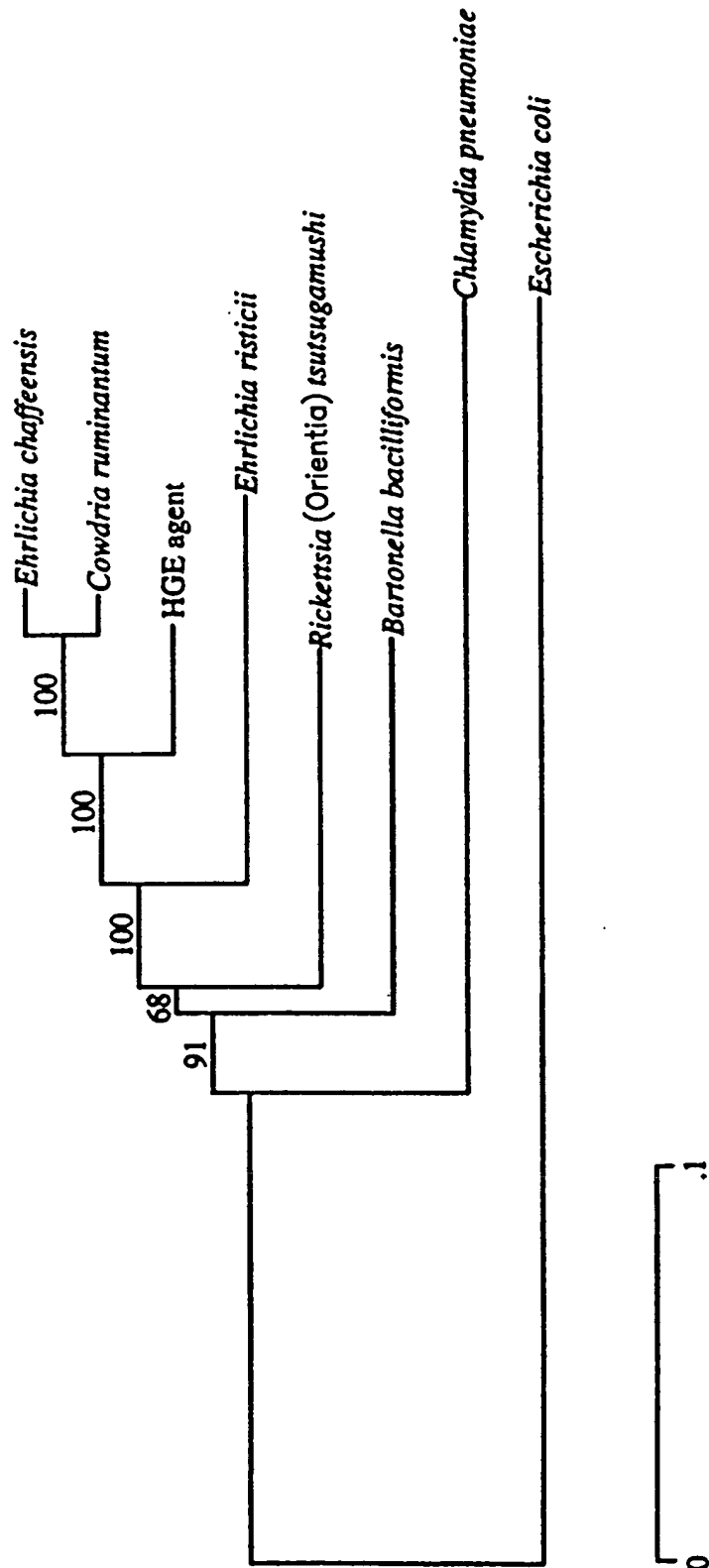


FIG. 3B

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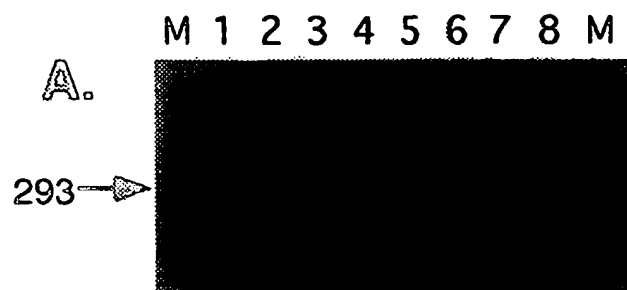


FIG. 4A

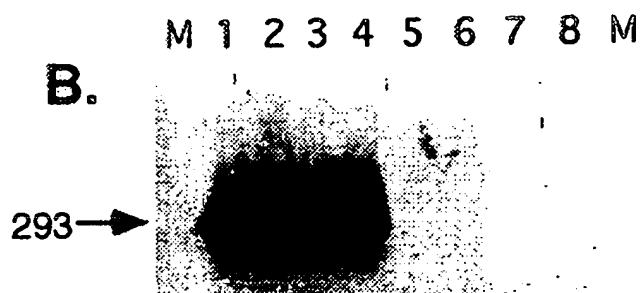


FIG. 4B

SUBSTITUTE SHEET (RULE 26)

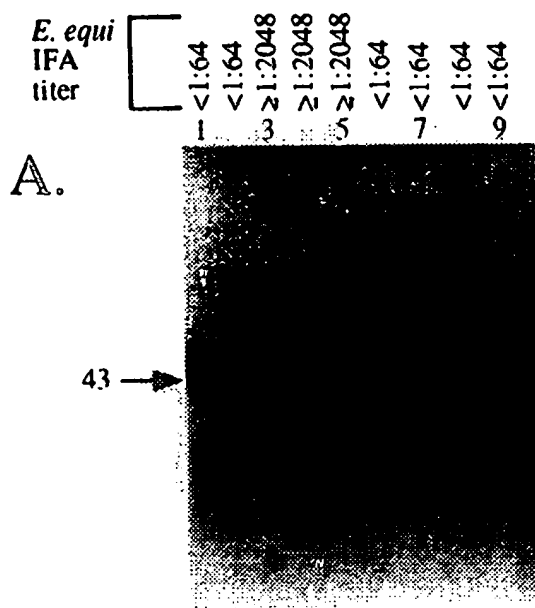


FIG. 5A

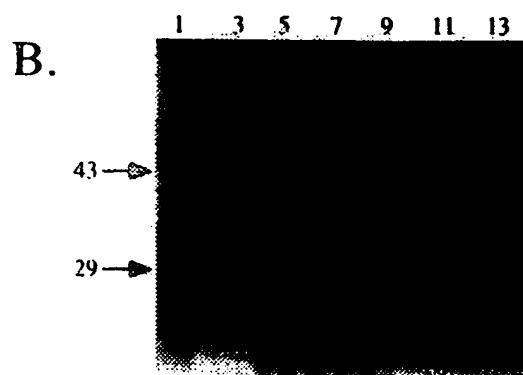


FIG. 5B

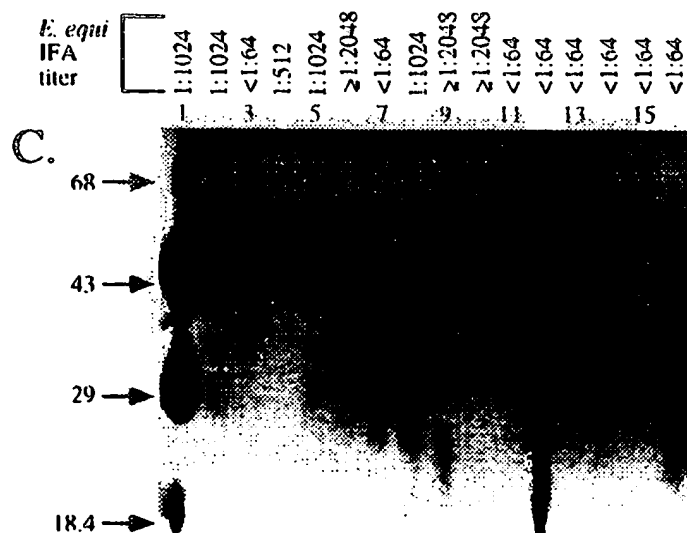


FIG. 5C

INTERNATIONAL SEARCH REPORT

In national Application No
PCT/US 98/05159

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C07K14/29 A61K39/02 C07K16/12 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUMNER J W ET AL: "Ehrlichia chaffensis expresses an immunoreactive protein homologous to the Escherichia coli GroEL protein" INFECTION AND IMMUNITY, vol. 61, no. 8, August 1993, pages 3536-3539, XP002071899 cited in the application see the whole document ---	1-5,9, 12,14, 17,18, 20, 22-27, 32,33
X	YU X -J ET AL: "Cloning and sequencing of the gene for a 120-kDa immunodominant protein of Ehrlichia chaffeensis" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES, vol. 184, no. 2, 15 January 1997, page 149-154 XP004093317 see the whole document ---	1,5,8,9, 11,12
-/--		

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

17 July 1998

Date of mailing of the international search report

30/07/1998

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Oderwald, H

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 98/05159

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	KOLBERT C P ET AL: "Evidence for human granulocytic ehrlichiosis in patients with suspected lyme disease" 95TH GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON USA, MAY 21-25, 1995. ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 95, 1995, page 36 XP002071900 see abstract C-204 see the whole document ---	1,20, 22-27
O,X	ASANOVICH K M ET AL: "PARTIAL CHARACTERIZATION OF CLONED GENES ENCODING IMMUNOREACTIVE PROTEINS OF EHRILICHIA EQUI AND THE AGENT OF HUMAN GRANULOCYTIC EHRILICHIOSIS (HGE)" ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, 19 May 1996, page 22 XP002058855 see abstract D-22 see the whole document ---	1,5,9, 12,14, 17,18,20
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